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Long-term feeding of genetically modified maize (MON810) – Metabolism of recombinant DNA and the novel protein in the dairy cow

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Für meine Mutter…

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Abbreviations

%	percent
Bt	***B**acillus **t**huringiensis*
CCα	decision limit
CCβ	detection capability
Ct	**c**ycle **t**hreshold (or Crossing Point)
CV	**c**oefficient of **v**ariation
CTAB	**c**etyl**t**rimethyl**a**mmonium**b**romide
DM	**d**ry **m**atter
FAO	**F**ood and **A**griculture **O**rganization of the United Nations
GIT	**g**astro**i**ntestinal **t**ract
gm	**g**enetically **m**odified
LOD	**l**imit **o**f **d**etection
OD	**o**ptical **d**ensity
PCR	**p**olymerase **c**hain **r**eaction
PTMR	**p**artial **t**otal **m**ixed **r**ation
qPCR	**q**uantitative real-time **PCR**
SEM	**s**tandard **e**rror of the **m**ean

Abstract

Genetically modified maize (MON810) is altered by insertion of the *cry1Ab* gene into the plant genome encoding for the Cry1Ab protein to gain resistance against one of the major pests, the European Corn Borer (*Ostrinia nubilalis*). As maize is commonly used as an animal feed, several short-term studies had been conducted on potential effects of feeding MON810 to livestock. So far, no long-term studies had been performed. Therefore, 36 lactating Fleckvieh cows were separated into two groups and fed on rations containing either genetically modified maize or the non-transgenic variety for 25 months. Feces, milk and blood samples were taken monthly. Additionally, feed samples were taken weekly, whereas urine samples were taken bimonthly.

Aim of this study was to investigate the fate of recombinant DNA and novel protein after feed intake, and analysis of the degradation pattern of the Cry1Ab protein in the gastrointestinal tract. Therefore, sensitive and specific assays were established and optimized to trace the *cry1Ab* DNA and the Cry1Ab protein in samples of the long-term experiment. A 206bp fragment of the recombinant *cry1Ab* DNA was detected by means of qualitative PCR, whereas short DNA-fragments of the bovine *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*, 354 bp) and the chloroplast gene *ribulose-1,5-bisphosphate carboxylase/oxygenase* (*rubisco*, 173 bp) were used as positive extraction and PCR controls. Additionally, feed and milk samples were analyzed using quantitative real-time PCR. In order to establish an enzyme-linked immunosorbent assay (ELISA) for the detection of immunoactive fragments of the Cry1Ab protein, highly specific polyclonal antibodies were raised in rabbits and maturated, before isolation by means of immuno affinity purification at high stringency. The assays were validated according to the European Commission Decision 2002/657/EC and the “Minimum Performance Requirements for Analytical Methods of GMO Testing” (published by the European Network of GMO Laboratories (ENGL)).

In maize silage, the DNA could only be detected in two samples and the Cry1Ab protein was only detected in lower concentrations, which is due to a rapid degradation of DNA and protein during the ensiling process. In contrast, all transgenic maize kernels and all transgenic maize cobs samples contained the *cry1Ab* DNA and the Cry1Ab protein was present. Hence, in all samples of the transgenic partial total mixed ration, the *cry1Ab* DNA and the novel protein were detected. The mean intake of cows in the group fed transgenic maize amounted to 6.0 mg Cry1Ab protein per cow daily. Non-transgenic feed samples were free of *cry1Ab* DNA and Cry1Ab protein. The recombinant DNA was not detected in feces samples, however, feces samples of cows fed transgenic feedstuff were found positive for the Cry1Ab protein. In blood, urine and milk, neither the *cry1Ab* DNA, nor the Cry1Ab protein could be detected. Investigations revealed a rapid degradation of recombinant *cry1Ab* DNA and the novel protein after feed intake in the bovine gastrointestinal tract. The Cry1Ab protein underlies a faster degradation process compared to other feed proteins. Milk from cows fed

genetically modified maize does not differ in any of the tested parameters from milk from cows fed conventional, not genetically modified maize.

Zusammenfassung

Mais (MON810) wurde durch die Integration des *cry1Ab*-Gens, das für das Cry1Ab-Protein kodiert, gentechnisch verändert (gv). Dieses Protein vermittelt eine Resistenz gegenüber dem bedeutendsten Maisschädling, dem Maiszünsler (*Ostrinia nubilalis*). Da dieser Mais auch als Futtermais für Tiere eingesetzt wird, wurden zahlreiche Kurzzeitstudien durchgeführt, um potentielle Effekte von MON810 auf Nutztiere zu untersuchen. Allerdings gab es bisher keine Studien über einen längeren Zeitraum beim Nutztier. Aus diesem Grund wurde im Rahmen dieser Dissertation eine Langzeitstudie mit 36 laktierenden Fleckviehkühen durchgeführt, die in zwei Gruppen mit je 18 Tieren aufgeteilt wurden. In dieser Studie, die 25 Monate dauerte, erhielt die Versuchsgruppe eine Futterration basierend auf gv Mais, die Kontrollgruppe erhielt Futter, das keinen gv Mais enthielt. Monatlich wurden Kot, Blut und Milchproben genommen, während Harnproben alle zwei Monate genommen wurden. Futterproben wurden im wöchentlichen Rhythmus gezogen.

Ziel dieser Studie war es den Verbleib der transgenen DNA und des rekombinanten Proteins, sowie das Abbauverhalten des Cry1Ab-Proteins im Verdauungstrakt zu untersuchen. Zu diesem Zweck wurden neue und sensitive Analysetechniken etabliert und optimiert, um validierte Aussagen über den Verbleib der rekombinanten DNA und des Cry1Ab-Proteins in den gewonnenen Proben des Langzeitversuchs treffen zu können. Alle Proben wurden mittels Polymerasekettenreaktion (PCR) auf ein 206bp Fragment der rekombinanten *cry1Ab*-DNA untersucht. Kurze DNA-Fragmente der bovinen *glycerinaldehyd-3-phosphat-dehydrogenase* (*GAPDH*, 354bp) und des Chloroplasten-Gens *ribulose-1,5-bisphosphat-carboxylase/-oxygenase* (*rubisco*, 173bp) dienten als Kontrollen der erfolgreichen Extraktion und PCR. Futter- und Milchproben wurden zusätzlich mittels quantitativer real-time PCR analysiert. Für die Etablierung eines enzyme-linked immunosorbent assay (ELISA) zur Detektion immunoaktiver Fragmente des Cry1Ab-Proteins wurden polyklonale Antikörper in Kaninchen generiert, maturiert und mittels Immunoaffinitätsaufreinigung bei hoher Stringenz isoliert. Die Assays wurden nach der Richtlinie 2002/657/EC der Europäischen Kommission und den „Minimum Performance Requirements for Analytical Methods of GMO Testing“ des European Network of GMO Laboratories (ENGL) validiert.

In der Maissilage konnte die rekombinante DNA lediglich in zwei Proben und das Cry1Ab-Protein generell in geringer Konzentration detektiert werden, was in einem starken Abbau während des Silierungsprozesses begründet liegt. Im Gegensatz dazu waren alle transgenen Proben von Maiskörnern und Maiskobs positiv für das Cry1Ab-Protein und das *cry1Ab*-Gen. Daher wurden auch

in allen Proben der partiellen totalen Mischration das Cry1Ab-Protein und die rekombinante DNA nachgewiesen. Die mittlere tägliche Aufnahme des Cry1Ab-Proteins pro Kuh der transgen gefütterten Gruppe betrug 6,0 mg. Nicht gv Futtermittel waren frei von rekombinanter DNA und rekombinantem Protein. In Kotproben konnte das *cry1Ab*-Gen nicht amplifiziert werden, allerdings war das Cry1Ab-Protein in allen Kotproben von Kühen, die mit transgenem Mais gefüttert wurden, detektierbar. In Blut, Harn und Milch konnten weder die transgene DNA, noch das transgene Protein detektiert werden. Die Untersuchungen zeigten, dass DNA und Proteine nach der Futteraufnahme im bovinen Gastrointestinaltrakt rasch abgebaut werden. Im Vergleich zu anderen Proteinen im Futter unterliegt das Cry1Ab-Protein sogar einem schnelleren Abbau. Milch von Kühen, die mit gv Mais gefüttert wurden, unterscheidet sich in den gemessenen Parametern nicht von Milch jener Kühe, die konventionellen nicht-gv Mais erhielten.

Introduction

Genetically modified maize

Since commercialization in 1996, the global cultivation areas of genetically modified (gm) plants increased from 1.7 million hectares in 1996 to 125 million hectares in 2008 (James, 2008). Soybean, as an important source of vegetable oil and protein, remained the most prominent gm crop in 2008, followed by gm maize, gm cotton and gm rapeseed (Figure 1).

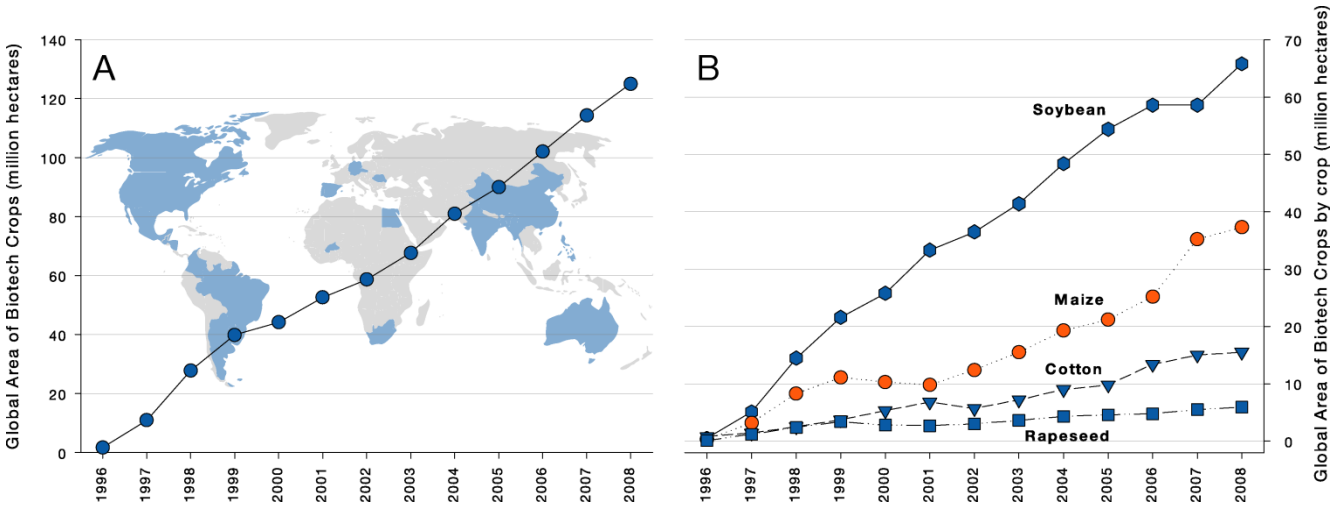

Figure 1: A) Cultivation areas with genetically modified plants, 1996 - 2008 (million hectares); B) Cultivation areas with genetically modified plants by crop, 1996 - 2008 (million hectares); (James, 2008)

Maize is a good source of carbohydrates, protein, vitamin B, and minerals and is therefore used for human consumption and as an animal feed. However, according to the estimations of the Food and Agriculture Organization of the United Nations (FAO), four percent of the yearly maize plants are destroyed by one of the major pests, the European Corn Borer (*Ostrinia nubilalis*).

Female moths lay 15-25 eggs on the underside of maize leaves. The eggs hatch within five to seven days and the larvae feed on developing leaves. After two weeks, the larvae bore into the stalk and excavate tunnels, resulting in water and nutrient translocation and reduced mechanical stability of the maize plant. Promoted by these damages, maize plants are often attacked by *Fusarium spp*., which produces Mycotoxines like Deoxynivalenol or Zearalenone leading to severe health problems in humans and animals (Königs et al., 2007; Königs et al., 2008; Lioi et al., 2004; Luongo et al., 2008).

Bacillus thuringiensis

An ubiquitary soil bacterium, the *Bacillus thuringiensis* (Bt), was found to produce a protein, which after intake leads to death of the larvae. During sporulation, the bacterium produces crystalline inclusions, containing δ-endotoxins, also called crystal proteins (Cry proteins) (Höfte and Whiteley, 1989). These Cry proteins are activated by proteases in the alkalic midgut of the larvae. The active Cry protein consists of three domains: the first domain binds to receptors, while the second domain

induces the formation of pores in the midgut epithelium. The third domain stabilizes the protein-receptor-complex and protects the other domains from cleavage by proteases. An influx of water along with ions results in cell swelling and lysis (English and Slatin, 1992). Furthermore, the efflux of intestinal content leads to sepsis and the formed pores are also portals of entry for bacteria and viruses leading to infections. As a result, the insect dies within several hours (Bravo et al., 2007; de Maagd et al., 2001; Schnepf et al., 1998).

Since 1964, Bt biologics have been used for biological pest control to reduce infestations by the European Corn Borer, even in organic farming. However, Bt biologics are only effective in the short period after hatching of the larvae and before they bore into the stalk. Therefore, along with Bt biologics, other management methods like chemical insecticides and biological enemies (*Trichogramma spec*.) are commonly used to fight the European Corn Borer (Figure 2). However, except for the insecticides, which are controversial discussed in the public, the effectiveness of the abatement methods is too low to prevent an infestation. Due to its high efficiency, the Bt concept was introduced to protect maize against the European Corn Borer. By use of genetic engineering, the *cry1Ab* gene from Bt was transferred into the maize genome (e.g. event MON810). After integration, this gene is regulated by a cauliflower mosaic virus (CaMV) 35S promoter resulting in a constant expression of Cry1Ab toxin, which is a truncated version of the original Bt protein and does not need an activation process. Therefore, MON810 is able to produce the novel protein to protect itself against the European Corn Borer.

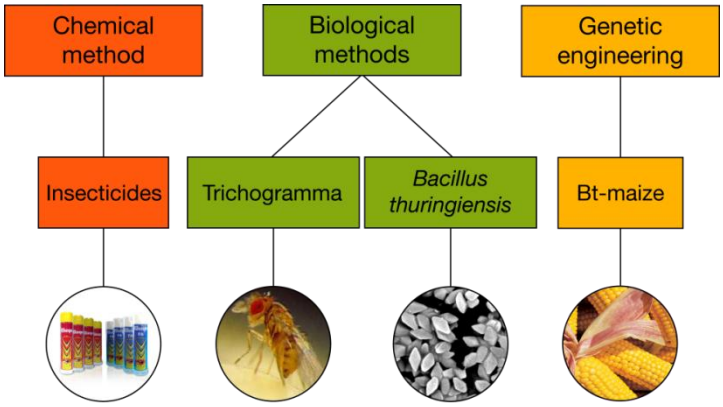

Figure 2: European Corn Borer – Management methods (based on Zellner, Bavarian State Research Center, IPZ, 2001)

Effects of feeding gm plants and persistence of novel DNA and protein

Since the commercial release of Bt maize, public concern has been expressed about potential effects of Cry1Ab on humans and animals, as Bt maize has been increasingly used for livestock and human consumption. In cell culture, no effects on cell proliferation and ATP level were observed after long-term exposure of Cry1Ab to ruminal epithelial cells (Bondzio et al., 2008) and also no effects on mammalian cell morphology, albumin secretion or LDH release were reported (Shimada et al., 2006a). Beyond that, several studies were conducted with livestock investigating effects of feeding MON810 on animal performance, milk yield, milk composition, feed intake and digestibility (Flachowsky et al., 2005). In pigs, feeding of gm maize had no effects on digestibility, feed intake and

body weight gain (Reuter et al., 2002b; Reuter et al., 2002a). Similar results were reported for ruminants. Studies were accomplished with dairy cows, steers and sheep, in which no significant differences in dry matter intake, weight gain, digestibility, milk yield and milk composition were obtained after feeding gm varieties (Donkin et al., 2003; Grant et al., 2003; Ipharraguerre et al., 2003; Yonemochi et al., 2003).

Another focus of recent short-term studies was the fate and the potential transfer of recombinant DNA and protein after intake of gm feed. After harvest, the amount of DNA and protein is reduced due to autolytic processes and microbial activity (Flachowsky et al., 2005). As a result of microbial enzymatic processes in the digestive tract and gastric acid, DNA and DNA fragments are further degraded (Alexander et al., 2002; Duggan et al., 2000; Sharma et al., 2004). A rapid degradation of DNA reduces the possibility of a transfer through the Peyer's Patches into the blood stream (Schubbert et al., 1997), however, small fragments of non-transgenic plant DNA were found in several tissues of broilers (Einspanier et al., 2001). Results of studies investigating the persistence of novel DNA and endogenous plant DNA in dairy cows also confirm the findings, that a transfer of plant DNA into the body is existent (Phipps et al., 2003). Up to now, transgenic DNA has not been detected in milk or blood of cows fed gm feed (Calsamiglia et al., 2007; Castillo et al., 2004). In contrary, in milk from the Italian market, recombinant DNA was detected, which the authors suspected to be caused by contamination (Agodi et al., 2006). Given that skim milk is preferably used for DNA extraction from milk, validated extraction and quantification methods for recombinant DNA from whole milk are scarce. Concerning the protein, microbial activity in the rumen leads to a rapid degradation to ammonia, nevertheless, some proteins pass through the rumen and are degraded in the small intestine. Therefore, immunoactive fragments can also be found in the feces (Einspanier et al., 2004) and in contents of the gastrointestinal tract (GIT) (Chowdhury et al., 2003b) of cows fed gm feed. In contrary, no recombinant protein was detected in milk after feeding gm plants (Calsamiglia et al., 2003; Yonemochi et al., 2003). It would be very unlikely for foreign DNA or any expressed protein of any plant gene to be found intact in blood or food of animal origin. However, only scare information is available on the persistence and the degradation pattern of Cry1Ab in dairy cows and no long-term experiments on feeding gm maize were accomplished, yet. Therefore, further studies and long-term experiments are needed to deal with the public concern expressed, regarding potential effects of gm plants on livestock and humans. In order to trace minute amounts of recombinant DNA or the Cry1Ab protein in feed, feces, blood, urine and whole milk, highly specific and sensitive extraction and detection methods are needed. These methods also need to be validated according to the existing guidelines for GMO testing.

Aim of the study

Given that only short-term studies were accomplished, to trace a potential transfer of recombinant DNA and protein during the metabolism of dairy cows, it was the aim of this study to investigate the persistence of *cry1Ab* DNA and the novel protein after feed intake in blood, feces, urine and milk of cows fed genetically modified maize (MON810) in comparison to cows fed non-transgenic maize (Figure 3). An important aspect of this work was to develop and optimize DNA and protein detection and quantification methods, and to validate these methods according to existing guidelines for GMO testing. As skim milk has been preferably used to analyze the fate of recombinant DNA so far, a DNA extraction and quantification procedure for whole milk shall be developed and validated. Further, the degradation pattern of the Cry1Ab protein in feed, feces and digesta contents of the gastrointestinal tract shall be analyzed by use of a very sensitive enzyme linked immunosorbent assay and an immunoblot.

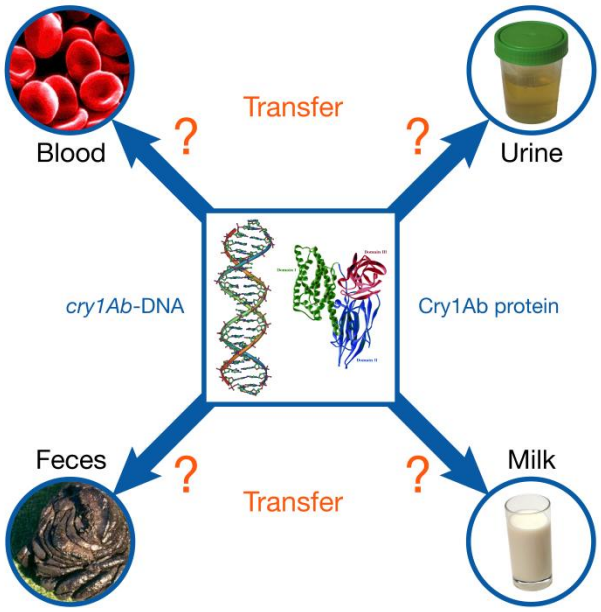

Figure 3: Schematic presentation of the study aim

Materials and Methods

Study design

A 25-months feeding study was conducted on 36 lactating Bavarian Fleckvieh cows, housed at the Bavarian State Research Center (LfL, Grub, Germany). The cows were separated into two groups and fed on diets containing either gm maize (MON810, target group, N=18, nine primiparous, nine multiparous) or the non-transgenic variety (control group, N=18, nine primiparous, nine multiparous). The daily feed ration contained a partial total mixed ration (PTMR) as shown in Table 1. Concentrates were offered above a milk yield of 22kg d^{-1}. Feed and water were offered ad libitum. The mean milk yield was 24.5 ± 7.2 kg d^{-1} (Guertler et al., 2009b; Steinke et al., 2009). Cows with a milk yield below 18 kg per day were fed a PTMR mixed with 27.7 % straw, whereas the dry ration consisted of a PTMR mixed with 35.7 % straw. The mean daily dry matter intake was 18.6 ± 3.6 kg. Nine cows of each group were replaced with heifers due to illness or infertility. Equivalence of the feed was achieved by feeding gm maize and a non-gm varieties with similar nutrient composition and energy content (Table 2) (Steinke et al., 2009). The GM maize and the non-transgenic variety had been cultivated and harvested in 2004, 2005 and 2006 under similar agronomic conditions at the Bavarian State Research Center for Agriculture (Germany). The study was performed under the approval of the Bavarian State Research Center (LfL) institutional animal care and use committee.

Table 1: Feed composition of the partial total mixed ration (Guertler et al., 2009a)

Component	Ratio (DM)
Maize silage[1]	41.9%
Maize cobs[1]	21.2%
Grass silage	11.0%
Straw	5.9%
Molasses	1.4%
Concentrates • Rapeseed meal • Maize kernels • Mineral mixture • Urea	18.6% • 51.1% • 41.2% • 5.3% • 2.4%

[1]Ingredients contained 100% MON810 in gm ration or conventional maize in non-gm ration

Table 2: Nutrient and energy content of non-gm maize and MON810 maize components (g/kg dry matter (DM)) (Steinke et al., 2009)

Constituent	Silage		Kernels		Cobs	
	non-gm maize	gm maize	non-gm maize	gm maize	non-gm maize	gm maize
Dry matter (g/kg)	361	351	885	895	905	907
Crude ash	28	29	15	15	33	32
Crude protein	80	84	102	98	82	83
Crude fat	31	28	41	40	25	30
Crude fiber	176	190	23	21	170	164
ADF[a]	188	229	27	24	193	195
NDF[b]	370	402	105	94	434	393
Net energy (MJ/kg DM)	6.74	6.67	8.75	8.81	6.99	7.01

[a] ADF = acid detergent fiber, [b] NDF = neutral detergent fiber

Sampling

Representative feed samples (maize kernels, maize cobs, maize silage and PTMR) were taken weekly and samples of four experimental weeks were pooled for analysis. Milk samples were taken monthly before milking in the morning at 06:00, blood and feces samples were taken monthly after milking, whereas spontaneous urine samples were taken bimonthly. The sampling scheme is depicted in Figure 4. All samples were stored at -20°C until used for DNA and protein analysis.

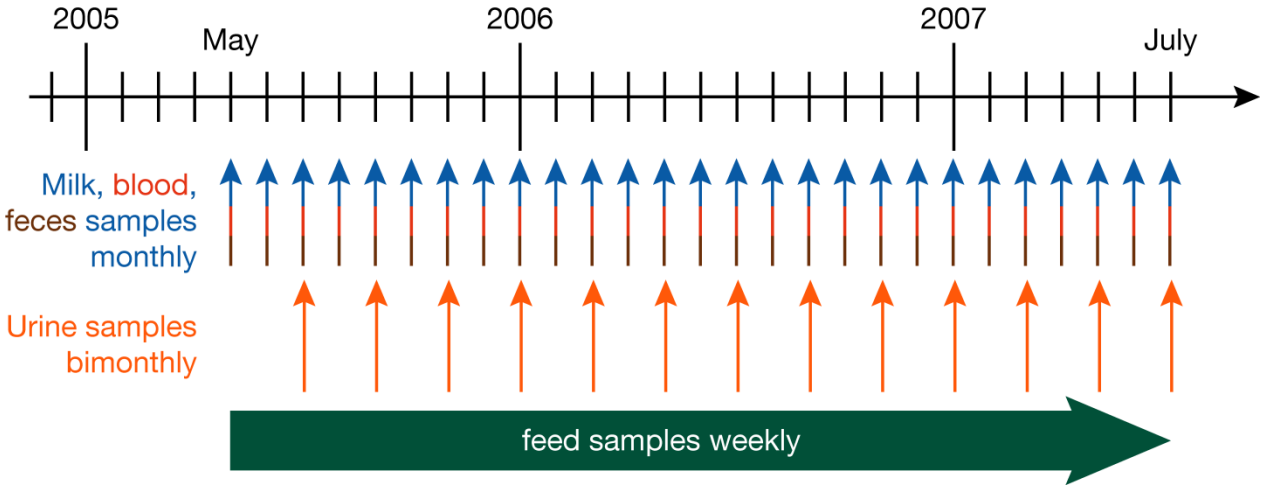

Figure 4: Sampling scheme of the long-term feeding experiment

DNA analysis

DNA extraction

For DNA extraction from feed and feces, a Cetyltrimethylammoniumbromide (CTAB)-based extraction protocol (Swiss Food Manual, 2004) was optimized and used as described in detail elsewhere (Guertler et al., 2009b). DNA from blood and urine were isolated using the commercial Invisorb Blood Universal Kit (Invitek, Germany) and PeqGold Trifast (Peqlab, Germany), respectively (Guertler et al., 2009b). An extraction method published by the Federal Office of Public Health (Swiss Food Manual, 2004) was optimized for DNA extraction from whole milk (Guertler et al., 2009a). Finally, DNA concentrations of all samples were determined by measuring the optical density (OD) at 260 nm and the ratio 260/280nm was used for DNA integrity determination (Biophotometer, Eppendorf, Germany). The recovery rate of each extraction procedure was determined by spiking samples with predefined copy numbers of *cry1Ab* DNA, followed by re-extraction and quantitative or qualitative analysis of the DNA.

Polymerase chain reaction

Small fragments of the bovine *glyceraldehyde-3-phosphate dehydrogenase* gene (*GAPDH*, 354 bp, Accession No. NM_001034034) were used as a positive extraction control for bovine DNA. GAPDH

plays a pivotal role in the glycolysis, catalyzing the conversion of glyceraldehyde 3-phosphate into D-glycerate-1,3-bisphosphate. As this is the crucial step in the glycolysis, the genes, encoding for GAPDH, are highly expressed multi copy genes and therefore are suitable as control genes to verify a successful DNA extraction from bovine matrices. The chloroplast gene *ribulose-1,5-bisphosphate carboxylase/oxygenase* (*rubisco*, 173 bp, Accession No. X86563) was used as positive extraction control to verify a successful DNA extraction from plant material. Ribulose-1,5-bisphosphate carboxylase/oxygenase is an enzyme that catalyzes the carboxylation or oxygenation of ribulose-1,5-bisphosphate in the Calvin cycle. It is the most abundant protein on earth (Dhingra et al., 2004) and its gene can therefore be used a positive extraction control for plant DNA. A 206 bp fragment of *cry1Ab* DNA (Accession No. AY326434) was amplified to trace the recombinant DNA incorporated in GM maize MON810. All primer pairs are listed in Table 3. The polymerase chain reactions (PCR) were performed as described in detail elsewhere (Guertler et al., 2007; Guertler et al., 2009a).

Table 3: Primer sequences used for amplification of small fragments of bovine *GAPDH*, maize *rubisco* and recombinant *cry1Ab*

Gene	Primer forward	Primer reverse	Product size
GAPDH	5'-ATCACTGCCACCCAGAAGAC-3'	5'-CCCAGCATCGAAGGTAGAAG-3'	354 bp
rubisco	5'-AGCTAATCGTGTGGCTTTAGAAGCC-3'	5'-TGGTATCCATCGCTTTGAAACCA-3'	173 bp
cry1Ab	5'-CCTGGAGAACTTCGACGGTA-3'	5'-TCGTGCCGTAGAGAGGAAAG-3'	206 bp

Amplicons were separated by gelelectrophoresis and visualized by staining with GelRed (Biotium, USA).

Quantitative real-time PCR

Copies of *cry1Ab* in feed and milk samples were quantified by means of quantitative real-time PCR (qPCR) using the LightCycler DNA Master SYBR green system (Roche Diagnostics, Germany) as previously described in detail (Guertler et al., 2009b; Guertler et al., 2009a). The qPCR MasterMix contained 1 µL LC FastStart DNA Master SYBR Green I (Roche Diagnostics, Germany), 4 pM of each primer, 3 mM $MgCl_2$, 1 µL target DNA (50 ng μL^{-1}) and water resulting in a final volume of 10 µL. PCR cycling conditions are depicted in Table 4. Each qPCR run included PCR grade water as a negative control and all samples were measured in duplicates.

Table 4: Cycling protocol for the quantification of a 206 bp fragment of *cry1Ab* using the LighCycler system (Roche Diagnostics, Germany)

	Cycles	Type	Target Temp	Time [sec.]	Acquisition Mode
Denaturation	1	Regular	95°C	600	None
Amplification	45	Quantification	95°C	15	None
			60°C	10	None
			72°C	25	None
			86°C	5	Single
Melting Curve	1	Melting Curve	95°C	5	None
			60°C	10	None
			99°C	0	Continuously
Cool	1	Regular	42°C	60	None

Additionally, a melting curve analysis was performed to verify the correct and selective amplification of the amplicon. Crossing Points (Ct) and the melting curve were obtained using the LightCycler 3.5.3 software (Roche Diagnostics, Germany). A dilution series of *cry1Ab* DNA (10^4 to 10 copies) was used to determine the limit of detection (LOD) for feed samples. Whole milk samples were spiked with different concentrations of *cry1Ab* (10^5 to 10^2 copies), followed by re-extraction of DNA. These spiked samples were used for LOD determination for milk samples by means of qPCR (Figure 5).

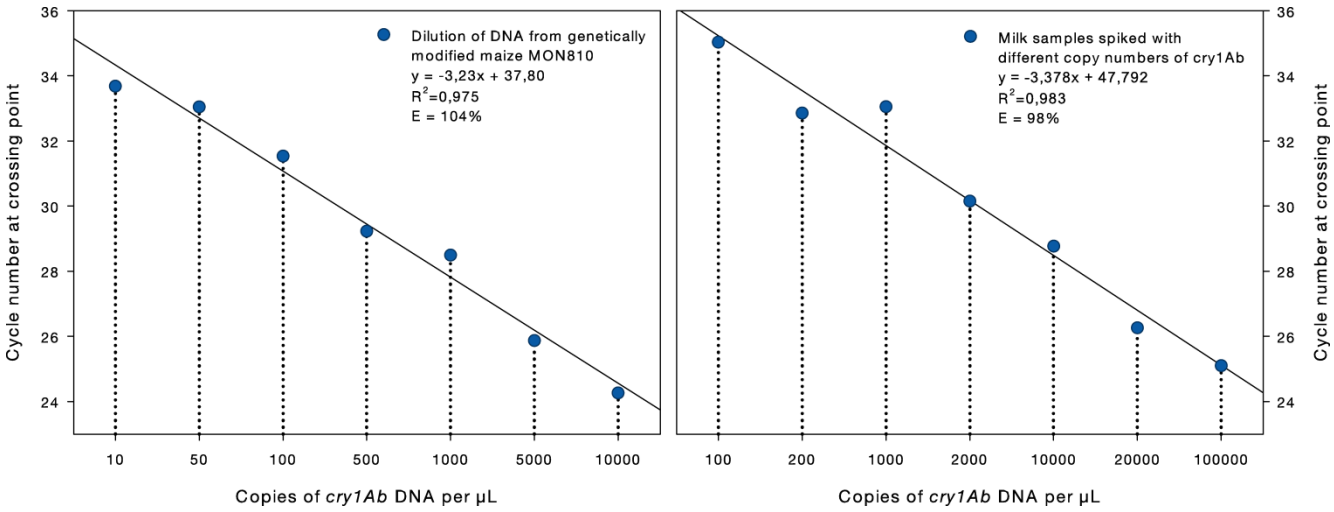

Figure 5: left panel: serial dilution of *cry1Ab* DNA obtained from maize kernels, showing linearity between 10 and 10^4 copies μL^{-1}; right panel: an exemplary standard curve of milk samples spiked with different copy numbers of cry1Ab as used in the qPCR. The standard curve shows the linearity between 100 and 100,000 copies μL^{-1}

Copy numbers were calculated by applying the following formula: (genomic DNA concentration in pg μL^{-1} × 6.0233 × 10^{23} copies mol^{-1})/(haploid maize genome (bp) × 660 × 10^{12}) (Mitchell et al., 2009). A genome size of 2.5×10^9 bp for the haploid maize genome was used (Arumuganathan and Earle, 1991).

Novel protein analysis

Detection of Cry1Ab protein fragments

For the development of a Cry1Ab detecting enzyme linked immunosorbent assay (ELISA), highly specific polyclonal antibodies were raised in crossbred rabbits through immunization and labeled with biotin after affinity purification (Paul et al., 2008). The Cry1Ab protein used for immunization and standard preparation was generously provided by Dr. William J. Moar (Auburn University, USA). The cross-reactivity with other Cry proteins was tested, underlining the specificity of the antibodies (Figure 6).

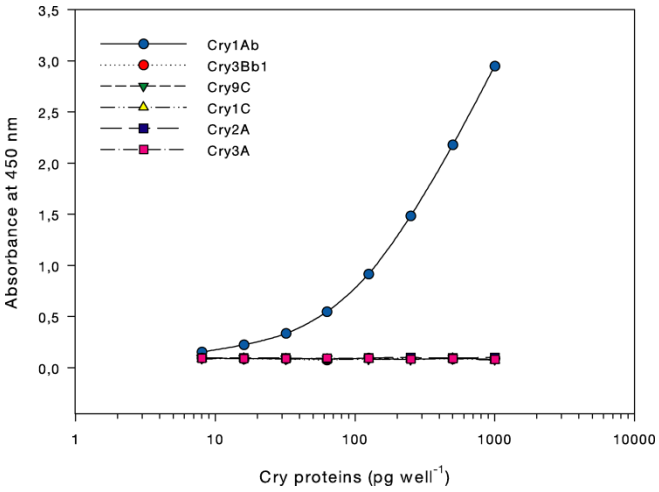

Figure 6: Test for cross-reactivity of the highly specific Cry1Ab antibody used in the ELISA with different Cry proteins (Paul et al., 2008)

For Cry1Ab protein analysis in feed (maize kernels, maize cobs, maize silage and PTMR) and feces, 100 mg of homogenized samples were used and extracted as previously described (Guertler et al., 2009b). 50µL sample extract was applied in the assay and the results are presented as ng of Cry1Ab protein g^{-1} feed or feces. An ELISA for Cry1Ab analysis in bovine blood was optimized and described in detail before (Paul et al., 2008). Urine samples were centrifuged at 4,000g and 4°C for 10 min and 40µL of the clear phase was used for Cry1Ab quantification (Guertler et al., 2009b). For Cry1Ab analysis in skim milk, an optimized and validated ELISA was applied (Guertler et al., 2009a). The data are presented in ng Cry1Ab protein mL^{-1} blood, urine or skim milk. All assays were validated as outlined in a previous publication (Guertler et al., 2009a; Paul et al., 2008).

Degradation pattern of the Cry1Ab protein

After slaughter at the end of the feeding trial, contents of rumen, abomasum, small intestine, large intestine and cecum were collected from six cows of each group to determine the amount and fragmentation pattern of Cry1Ab protein in the GIT digesta. All samples were stored at –80°C until analyzed for total protein and Cry1Ab protein as described elsewhere (Paul et al., 2009). Total protein concentration in each extract was measured by bicinchoninic acid (BCA) assay (Smith et al., 1985) using BSA as protein standard.

To monitor the fragmentation of Cry1Ab protein from MON810 in transgenic feed, feces and GIT digesta, an immunoblot analysis was performed (Paul et al., 2009).

Statistics and data analysis

In the qPCR, data were analyzed using the standard curve method as described in detail elsewhere (Guertler et al., 2009b; Guertler et al., 2009a). The assays were validated according to the "Minimum Performance Requirements for Analytical Methods of GMO Testing" (published by the European Network of GMO Laboratories (ENGL)). Analysis of three samples from the standard curve containing different copy numbers of *cry1Ab* (for feed 10,000, 500 and 50 copies μL^{-1}; for milk 10,000, 100 and 10 copies μL^{-1} after spiking) was performed to determine the intra- and interday coefficient of variation (CV). The PCR efficiency was calculated using following the formula: $E = [10^{-1/slope}-1] \times 100$.

Concerning the protein analysis, the decision limit ($CC\alpha$) and the detection capability ($CC\beta$) of the ELISA were determined according to the guidelines of the European Commission Decision 202/657/EC (European Commission, 2002) as described before (Paul et al., 2008). The following formulas were used to calculate the $CC\alpha$ and the $CC\beta$:

(1) $CC\alpha$ = (background noise level for Cry1Ab in blanks) × 3

(2) $CC\beta = CC\alpha + 1.64 \times$ standard deviation

The standard deviation was obtained using blank samples fortified at concentrations of $CC\alpha$. Data are presented as mean of all cows (target and control group) per month ± standard error of the mean (SEM).

To compare Cry1Ab protein concentrations in transgenic feed and feces, and total protein in the GIT digesta of cows fed non-transgenic or transgenic diets, a Student's t-test was used. A *P*-value below 0.05 was considered significant.

Results and Discussion

Feed

DNA. DNA extraction from feed resulted in high DNA concentrations with a DNA purity suitable for PCR analysis, as the OD 260/280nm ratios were between 1.60 and 1.98. In detail, DNA concentrations ranging from 42 to 970 ng DNA μL^{-1} for maize kernels, from 49 to 359 ng DNA μL^{-1} for maize cobs, from 41 to 163 ng DNA μL^{-1} for maize silage and from 38 to 197 ng DNA μL^{-1} for PTMR were achieved. All feed samples (maize kernels, maize cobs, maize silage and PTMR) underwent PCR analysis by amplification of a 173 bp fragment of *rubisco* and qPCR analysis by amplification of a 206 bp fragment of *cry1Ab*. A limit of detection (LOD) was determined at 37 copies of *cry1Ab* g^{-1} DM with a PCR efficiency of 104%. Mean intra- and inter-assay CVs of 0.15 (n=9) and 0.20 (n=3 six replicates each) underline the suitability of the DNA extraction and quantification procedure (Guertler et al., 2009b). Further, a melting curve analysis and subsequent sequence analysis ensured the specificity of the amplicon.

Protein. Validation of the matrix-matched ELISA revealed a good assay precision, illustrated by a mean intra-assay CV of 3.57 (n=8, three replicates each) and a mean inter-assay CV of 7.94 (n=8, three replicates each (Guertler et al., 2009b). Recovery rates and determined CCα and CCβ values are shown in Table 5.

Table 5: Validation of the matrix-matched ELISA for Cry1Ab protein detection in feed samples

	CCα [ng g^{-1} DM]	CCβ [ng g^{-1} DM]	Mean recovery rate [%]
Maize kernels	1.35	2.08	88.6
Maize cobs	1.32	1.65	74.7
Maize silage	4.6	5.61	76.5
PTMR	8.18	12.26	78.9

Feed samples. In all feed samples of the long-term study, a 173 bp fragment of the chloroplast gene *rubisco*, was detected using conventional PCR, underlining the suitability of the extraction method. Non-transgenic feed samples were tested negative for *cry1Ab* DNA or fragments of the Cry1Ab protein by means of qPCR and ELISA. Only in maize kernels, two sets of non-transgenic samples were found positive for Cry1Ab protein fragments, which might be due to sampling or post-sampling contamination. The recombinant DNA and immunoreactive fragments of the novel protein were detected in all transgenic feed samples, except of maize silage. Recombinant DNA was not present in maize silage, except of two sets of transgenic samples. This goes in line with the findings that the Cry1Ab protein was detected in transgenic maize silage in lower concentrations in comparison to the other maize components, which was reported to be caused by a rapid degradation of DNA and

protein throughout the ensiling process (Hupfer et al., 1999; Lutz et al., 2006). The range of transgenic components, detectable by means of qPCR and ELISA in feed samples, is depicted in Figure 7 and Figure 8. Data obtained for the recombinant DNA and the novel protein in feed samples are comparable concerning the particular feed components. This study revealed, that most of the Cry1Ab protein and *cry1Ab* DNA originate from maize cobs, followed by maize kernels and maize silage (Guertler et al., 2009b), which is in accordance to the statement that the Cry1Ab protein is expressed in leaves, tassels, kernels, silk tissue and the stalk (Horner et al., 2003; Hubert et al., 2008)). The highest Cry1Ab protein concentration was stated to be found in leaves, where it is up to 20 times higher than in kernels (Hubert et al., 2008). By the use of different maize components (kernels, cobs and silage), a mean daily intake of 6 mg Cry1Ab protein per cow was achieved.

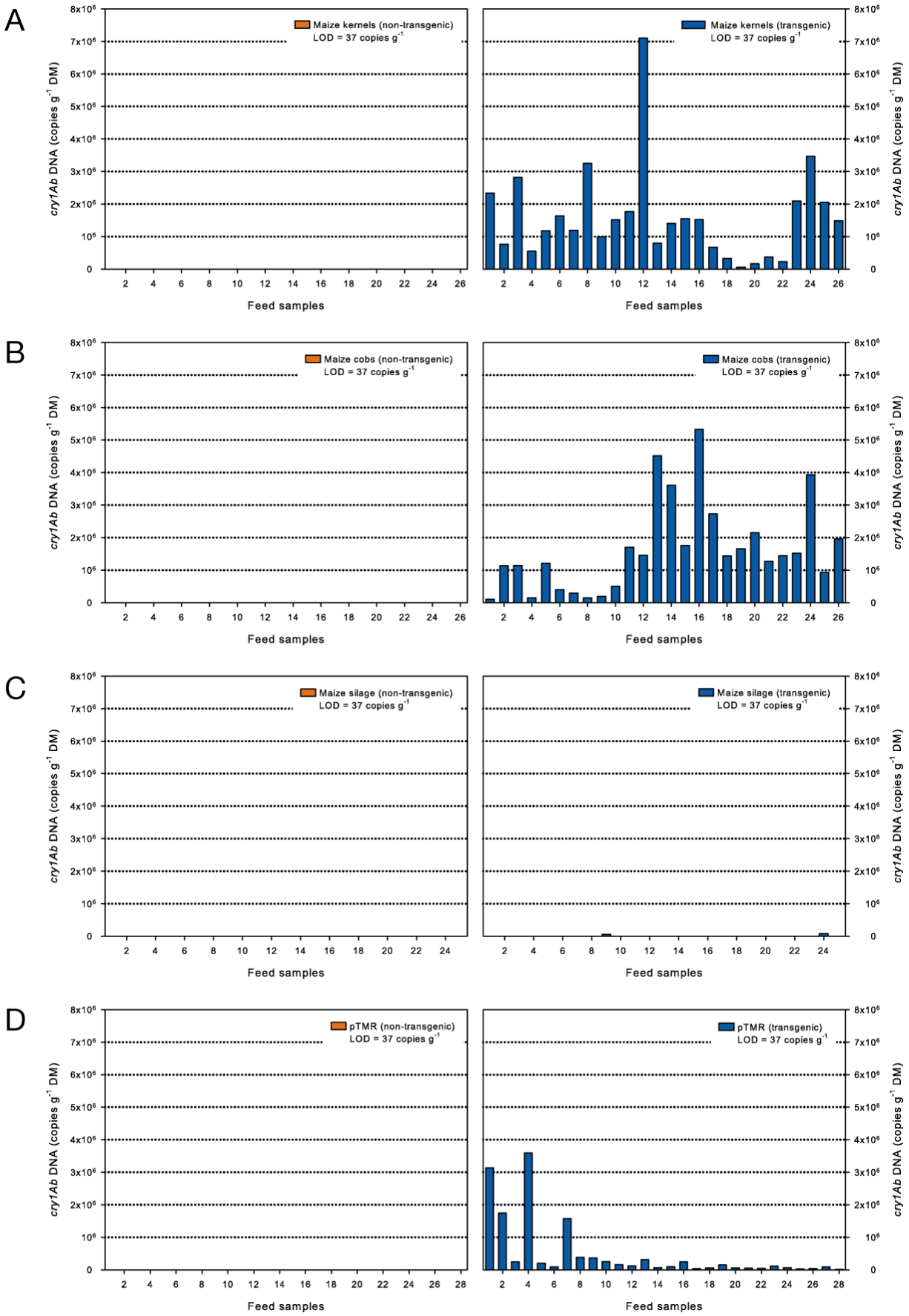

Figure 7: Quantification of a 206 bp fragment of *cry1Ab* by means of quantitative real-time PCR in A) maize kernels, B) maize cobs, C) maize silage and D) partial total mixed ration (**PTMR**); LOD = limit of detection

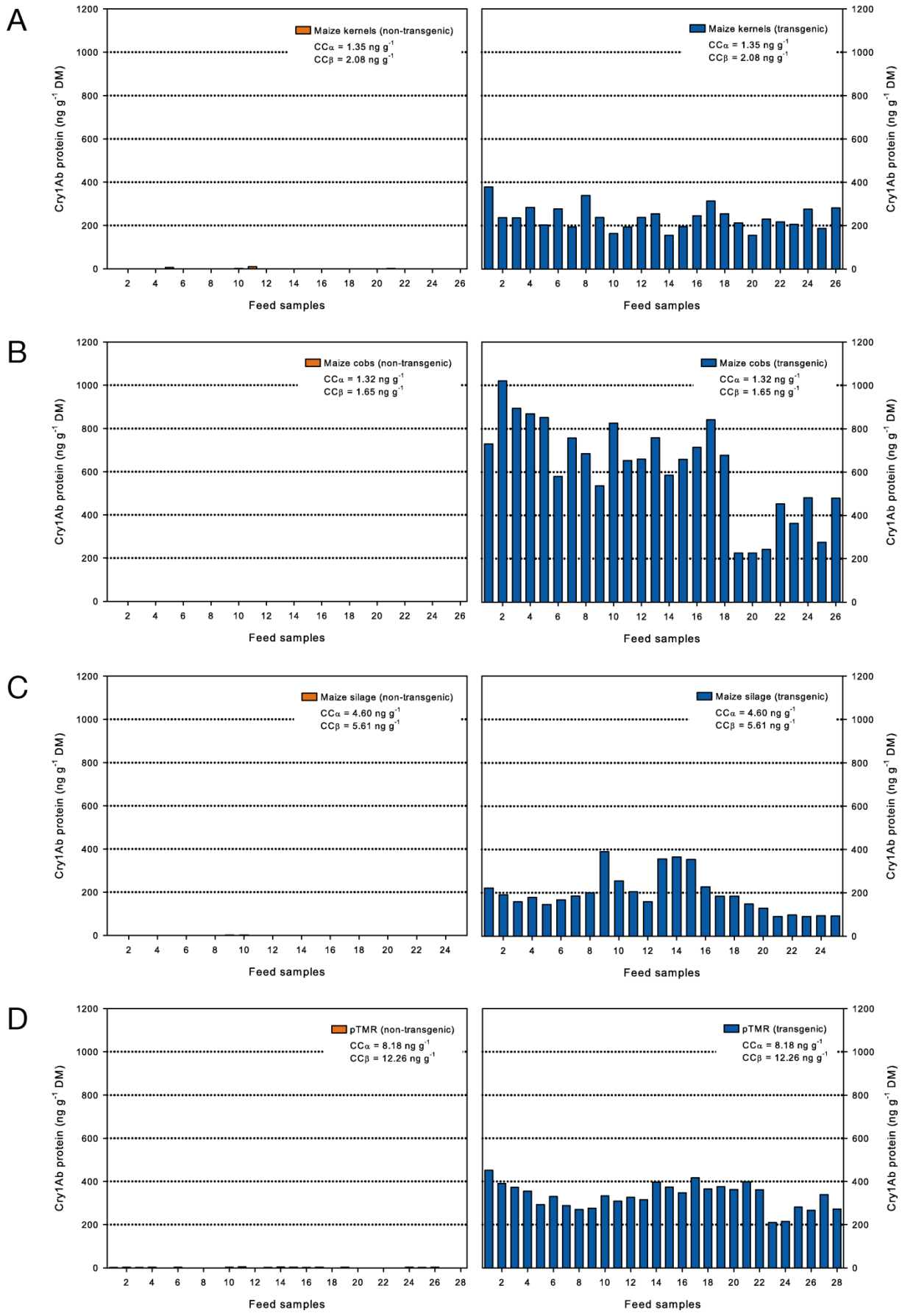

Figure 8: Quantification of the Cry1Ab protein using a matrix-matched ELISA; A) maize kernels, B) maize cobs, C) maize silage, D) partial total mixed ration (**PTMR**); CCα = decision limit; CCβ = detection capability

Feces

DNA. DNA extraction resulted in concentrations above 50 ng μL^{-1} and a good DNA purity, elucidated by 260/280 ratios between 1.6 and 1.8, underlining the suitability of the extraction method. In all feces samples, a 173 bp fragment of *rubisco* was detected, which served as a positive extraction control. By means of conventional PCR, a 206 bp fragment of *cry1Ab* was not amplified at a calculated LOD of 17,000 copies g^{-1} feces (wet weight), as shown in Figure 9.

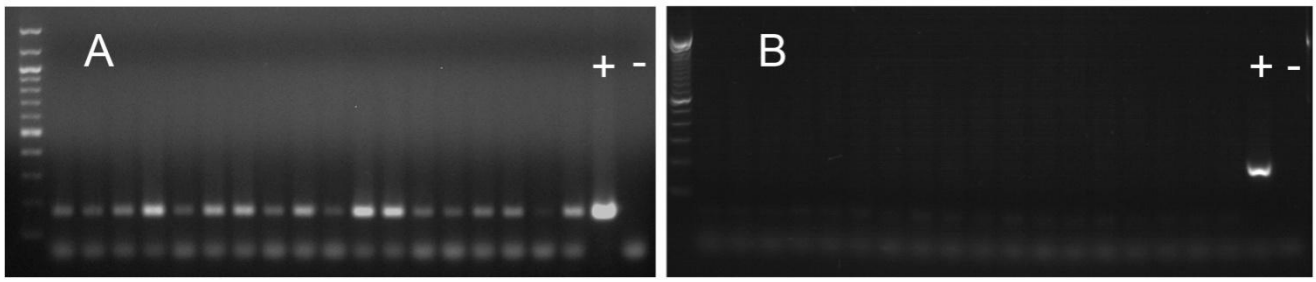

Figure 9: (A) Amplification of a 173 bp fragment of the maize specific *rubisco* gene as a positive extraction control in feces samples from cows fed GM maize; (B) amplification of a 206 bp fragment of the recombinant *cry1Ab* gene in feces samples from cows fed GM maize; (positive controls are marked with a "+", negative controls are marked with a "-")

Protein. Cry1Ab assay validation revealed a CCα of 1.21 ng g^{-1} feces (wet weight) and a CCβ of 2.00 ng g^{-1} feces (wet weight) (Guertler et al., 2009b)

Feces samples. Feces samples from cows fed non-gm maize were below the decision limit CCα, whereas samples from the target group ranged from 20 to 110 ng Cry1Ab protein g^{-1} feces (wet weight) (Figure 10). Previous studies go in line with these findings, as the novel protein was detected in feces of calves (Chowdhury et al., 2003b) and in the gastrointestinal content of pigs (Chowdhury et al., 2003a) fed genetically modified maize. The novel protein is not completely degraded in the bovine gastrointestinal tract due to a limited digestibility of maize crude protein (Romagnolo et al., 1994).

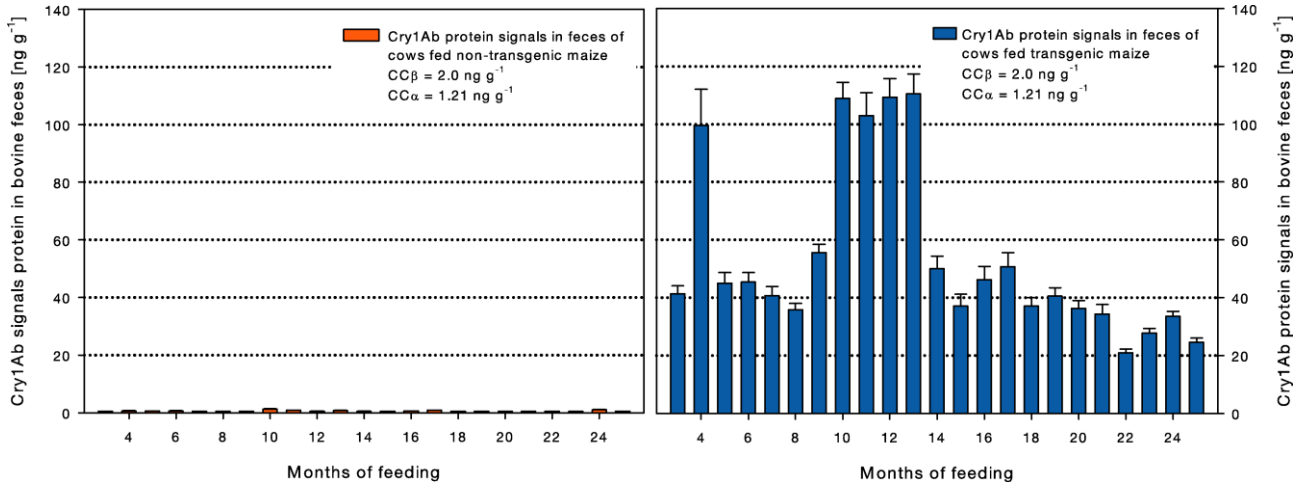

Figure 10: Signals for Cry1Ab protein in bovine feces from cows fed non-transgenic maize (left panel) or transgenic maize (right panel); CCα = decision limit; CCβ = detection capability; error bars indicate the standard error of the mean (SEM)

Blood

DNA. DNA isolation from bovine blood samples by use of the commercial Invisorb Blood Universal Kit resulted in concentrations between 40 and 800 ng ml^{-1}. OD ratios (260/280 nm) of 1.5 to 1.9 underline the purity of the DNA. A 354 bp fragment of the highly abundant *GAPDH* was analyzed in all samples, verifying the suitability of the extraction procedure. The suitability of *GAPDH* as a positive control for extraction from milk and blood is often described (Leutenegger et al., 2000; Robinson et al., 2007). Further, all samples were analyzed for an amplification of a 173 bp fragment of *rubisco* and a 206 bp fragment of the *cry1Ab* gene. The LOD of the recombinant *cry1Ab* gene was determined at 10^5 copies of *cry1Ab* ml^{-1} blood.

Protein. For Cry1Ab protein analysis, an optimized ELISA with a decision limit CCα of 1.53 ng ml^{-1} blood and a CCβ of 2.3 ng ml^{-1} blood was used. The recovery rate was between 89 and 106% (Paul et al., 2008).

Blood samples. In all blood samples of the long-term study, a 354 bp fragment of bovine GAPDH was amplified, whereas fragments of maize *rubisco* (173 bp) and the recombinant *cry1Ab* (206 bp) were absent (Figure 11). Although it is reported, that DNA fragments are able to cross the intestinal barrier into the blood stream (Alexander et al., 2007), a transfer of novel DNA from feed into blood was not shown (Einspanier et al., 2001; Nemeth et al., 2004; Phipps et al., 2003).

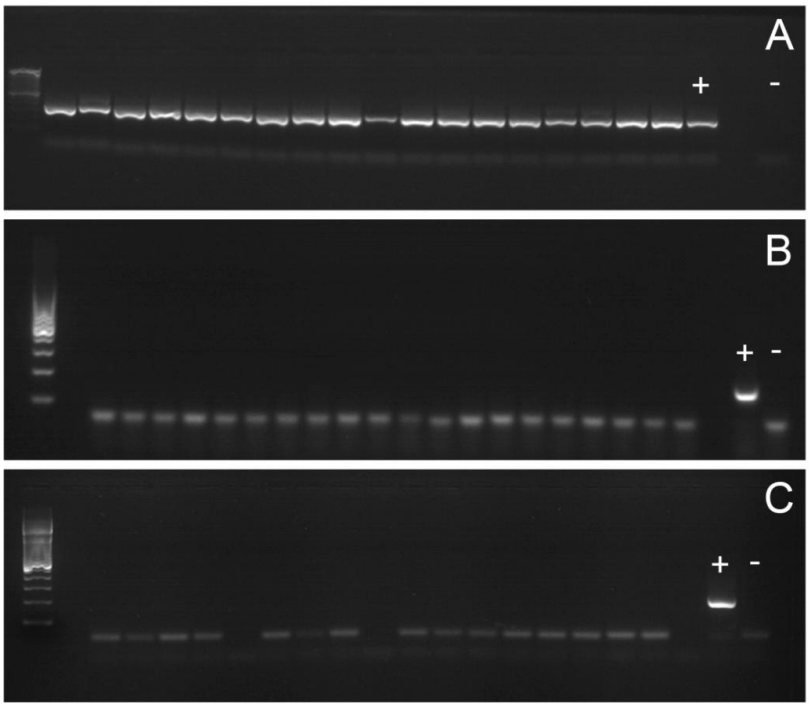

Figure 11: (A) Amplification of a 354bp fragment of *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) as a positive extraction control in blood samples of cows fed genetically modified maize; (B) amplification of a 173bp fragment of *rubisco*. The highly abundant chloroplast gene was not detected in blood samples; (C) a 206bp fragment of *cry1Ab* was not detected in blood samples of the long-term experiment. (positive controls are marked with a "+", negative controls are marked with a "-")

No difference between the control and the target group were observed for the Cry1Ab protein, as all ELISA values were below the CCα of 1.53 ng ml^{-1} (Figure 12). Recombinant DNA and the novel protein are rapidly degraded within the bovine gastrointestinal tract (Lutz et al., 2005; Wiedemann et al., 2006) and the low number of copies of *cry1Ab* would also impede the tracing of a possible transfer of recombinant DNA into the blood stream (Bertheau et al., 2009). So it is highly unlikely that intact DNA can pass the GIT and is absorbed via the Peyers' Patches (Schubbert et al., 1997). Further, the lack of Cry1Ab specific receptors in the bovine intestinal epithelium (Shimada et al., 2006a; Shimada et al., 2006b) and the lack of absorption mechanisms may hinder a potential transfer of Cry1Ab protein from the gut into the blood.

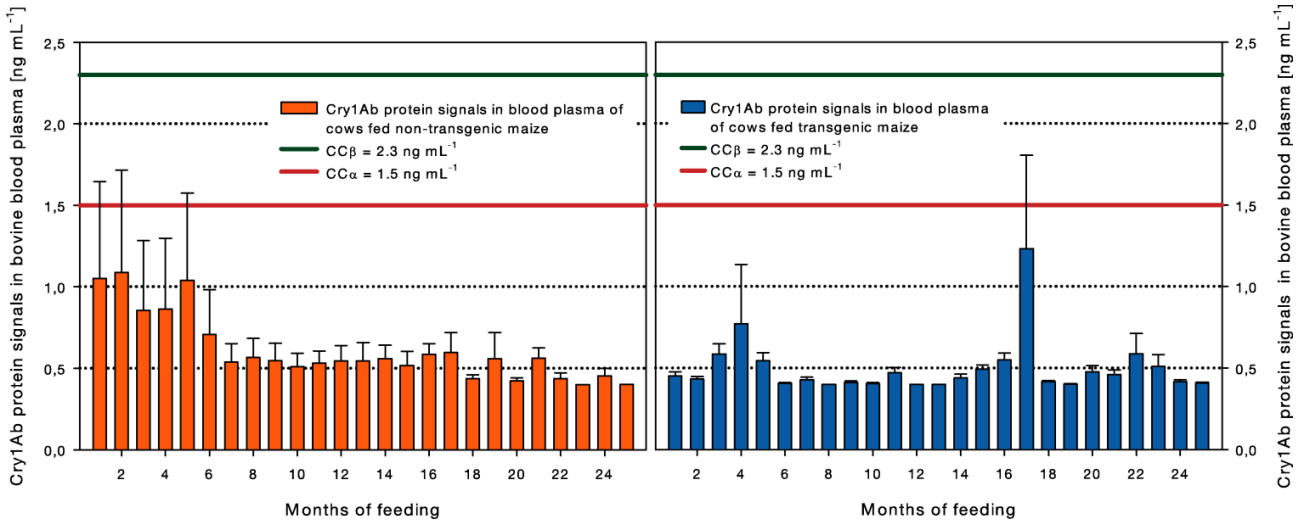

Figure 12: Background signals for Cry1Ab protein in bovine blood from cows fed non-transgenic maize (left panel) or transgenic maize (right panel); CCα = decision limit, marked with a red line; CCβ = detection capability, marked with a green line; error bars indicate the standard error of the mean (SEM)

Urine

DNA. DNA extraction from bovine urine samples resulted in low DNA concentrations between 6 ng µL^{-1} and 40 ng µL^{-1}. Ratios of 260/280 nm ranged from 1.3 to 1.7. As a positive extraction control, a 354 bp fragment of *GAPDH* was amplified.

Protein. Validation of the Cry1Ab ELISA revealed a CCα of 0.36 ng ml^{-1} and a CCβ of 0.3 ng ml^{-1} (Guertler et al., 2009b).

Urine samples. At a LOD of 23,000 copies mL^{-1}, no sample was found positive for a 206 bp fragment of *cry1Ab*. *GAPDH* was detected in most urine samples. The absence of *cry1Ab* in urine might be ascribed to the low genomic DNA concentrations. It is also stated, that urea inhibits DNA amplification by means of PCR (Khan et al., 1991). Cry1Ab protein analysis resulted in values below the CCα, except of one sample of the target group showing a protein fragment concentration of 1.59 ng mL^{-1}. This is most likely due to fecal contamination, also expressed by a high SEM of 0.297 (Figure 13).

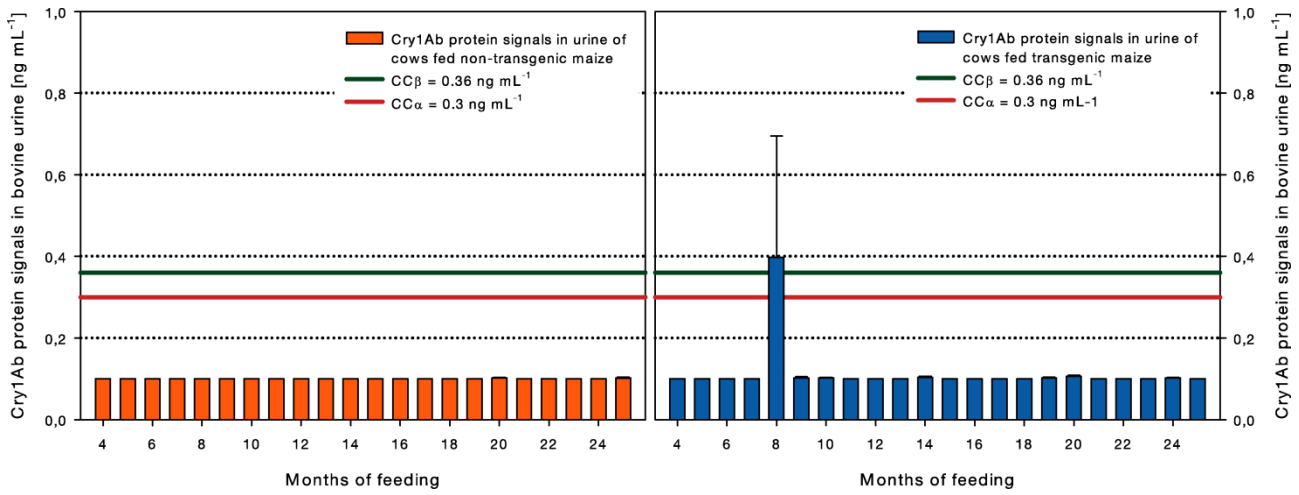

Figure 13: Background signals for Cry1Ab protein in bovine urine from cows fed non-transgenic maize (left panel) or transgenic maize (right panel); CCα = decision limit, marked with a red line; CCβ = detection capability, marked with a green line; error bars indicate the standard error of the mean (SEM)

Milk

DNA. Genomic DNA concentrations between 45 and 250 ng μL^{-1} and 260/280nm ratios between 0.9 to 1.8 were achieved using a guanidinhydrochloride-based DNA extraction method. The analytical range for *cry1Ab* DNA in whole milk was 10^2 to 10^5 copies μL^{-1} with a mean recovery rate of 84.9 % (n=3, six replicates each), an intra-assay CV of 0.15 % (n=9) and an inter-assay CV of 0.78 % (n=9, three replicates each).

Protein. A CCα of 0.25 ng mL^{-1} and a CCβ of 0.4 ng mL^{-1} were determined for Cry1Ab protein analysis. The recovery rate ranged from 88 to 104% (Guertler et al., 2009a).

Milk samples. A small fragment of *GAPDH* (354 bp) was amplified in all milk samples of the long-term study, underlining the suitability of the extraction method. However, fragments of *rubisco* (173 bp) and *cry1Ab* (206 bp) were not detected (Figure 14).

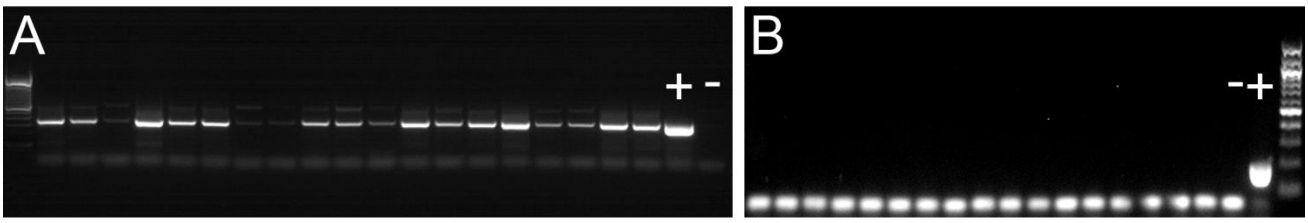

Figure 14: (A) Specific amplification of a 354bp fragment of *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) as a positive extraction control in milk samples of cows fed genetically modified maize; (B) amplification of a 206bp fragment of *cry1Ab* in milk samples of the long-term experiment. (positive controls are marked with a "+", negative controls are marked with a "-")

By applying qPCR, no milk sample was found above the LOD for *cry1Ab* of 100 copies μL^{-1}. Analysis of skim milk for immunoactive novel protein fragments using an ELISA resulted in values below the CCα, showing no differences between the control group and the target group (Figure 15). Previous studies confirm the absence of recombinant DNA or novel protein in milk (Calsamiglia et al., 2007;

Nemeth et al., 2004; Phipps et al., 2002; Phipps et al., 2003; Phipps et al., 2005), though, small fragments of recombinant DNA were detected in milk samples from the Italian market, which the authors suspected to be due to feed and fecal contamination during milking (Agodi et al., 2006).

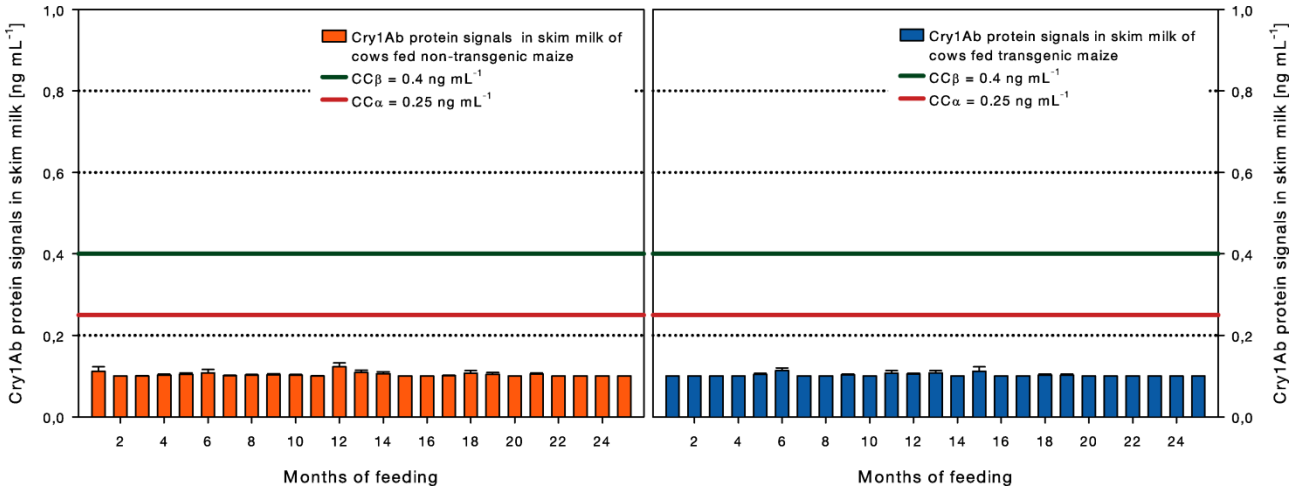

Figure 15: Background signals for Cry1Ab protein in bovine urine from cows fed non-transgenic maize (left panel) or transgenic maize (right panel); CCα = decision limit, marked with a red line; CCβ = detection capability, marked with a green line; error bars indicate the standard error of the mean (SEM)

Developments in Cry1Ab protein and *cry1Ab* DNA analysis

At the onset of this study, an ELISA, which had been validated according to the guidelines of the European Commission Decision 2002/657/EC for Cry1Ab protein detection, was not available. Hence, the development of new extraction and quantification methods for *cry1Ab* DNA and Cry1Ab protein analysis was in focus of this study. A sensitive and specific ELISA was established using specific, affinity purified antibodies. PCR and qPCR assays were developed for the analysis of the recombinant *cry1Ab* DNA. Additionally, all assays were validated by determining the decision limit and the detection capability of the ELISA, as well as the LOD for *cry1Ab* DNA quantification, which is summarized in Table 6.

Table 6: Decision limit (CCα) and detection capability (CCβ) of the Cry1Ab protein ELISA and limit of detection (LOD) for the *cry1Ab* DNA in feed, feces, blood, urine and milk

	CCα (Protein)	CCβ (Protein)	LOD (DNA)
Feed			
Maize kernels	1.35 ng g^{-1}	2.08 ng g^{-1}	37 copies g^{-1}
Maize cobs	1.32 ng g^{-1}	1.65 ng g^{-1}	37 copies g^{-1}
Maize silage	4.60 ng g^{-1}	5.61 ng g^{-1}	37 copies g^{-1}
PTMR	8.18 ng g^{-1}	12.26 ng g^{-1}	37 copies g^{-1}
Feces	1.2 ng mL^{-1}	2.0 ng mL^{-1}	1,7*10^4 copies g^{-1}
Blood	1.5 ng mL^{-1}	2.3 ng mL^{-1}	10^5 copies mL^{-1}
Urine	0.3 ng mL^{-1}	0.36 ng mL^{-1}	2,3*10^4 copies mL^{-1}
Milk	0.25 ng mL^{-1}	0.4 ng mL^{-1}	10^5 copies mL^{-1}

Protein degradation pattern

Quantification of Cry1Ab protein fragments in feed and feces revealed a decrease in content of immunoactive Cry1Ab protein fragments in relation to total protein. Compared to the initial Cry1Ab fraction of the total protein content in feed, the relative Cry1Ab fraction in feces from cows fed gm diets decreased to 44% ($p < 0,01$) (Paul et al., 2009) (Figure 16). The total protein concentration in feces of cows fed a gm-maize based ration were 50% of the initial levels of the total proteins. Therefore, in comparison to the total protein content, the Cry1Ab protein is expeditiously degraded. The corresponding samples of non-transgenic feed and feces were found below the assay decision limit.

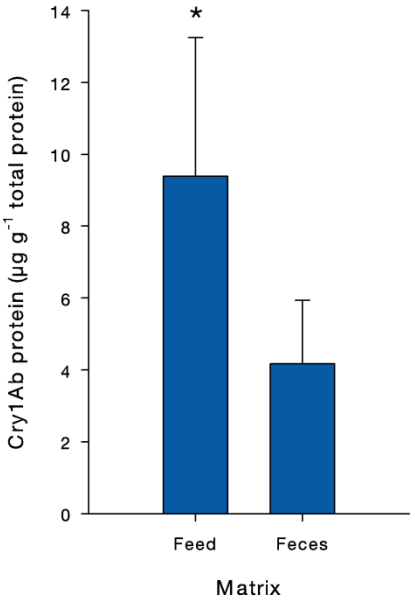

Figure 16: Content of immunoactive Cry1Ab protein fragments in relation to total protein in feed (MR) and feces of dairy cows fed a partial mixed ration diets containing transgenic maize
* indicates ($P < 0.01$).

Concentrations of immunoactive Cry1Ab protein fragments in analyzed digesta contents of the GIT were compared to corresponding total protein concentrations. In rumen, abomasum, small intestine, large intestine and cecum of cows fed transgenic diets, 3.84, 0.38, 0.83, 2.89 and 3.18 µg of Cry1Ab protein per g total protein were obtained (Figure 17).

In the rumen, the highest concentration of Cry1Ab fragments was observed, which is most probably due to large undigested feed particles, whereas the release of microbial proteins into the abomasum results in a decrease of the Cry1Ab protein total protein ratio in the abomasum. In comparison to the total protein concentration, the concentration of Cry1Ab fragments increases in subsequent segments of the GIT, which might be due to absorption of end products of the protein digestion in the small intestine (Paul et al., 2009). Similar degradation patterns were observed in previous studies (Einspanier et al., 2004; Lutz et al., 2005).

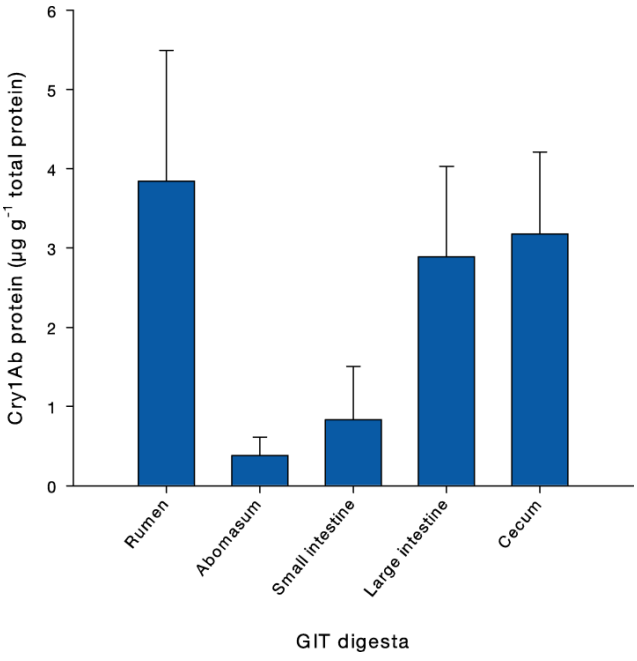

Figure 17: Relative Cry1Ab protein concentrations (± SD) in different GIT digesta for cows fed a partial total mixed ration diets containing transgenic maize.

By means of immunoblot analysis, GIT digesta and feces samples revealed a degradation of the Cry1Ab protein (65 KDa) into smaller immunoactive fragments of approximately 42, 34 and 17 kDa, whereas the 34 kDa fragment was the most prominent fragment. The 42 kDa fragment was only detected in feed samples (Figure 18). As these smaller fragments seem to be regular intermediates, it will be interesting whether these fragments show any biological activity. A time dependent fragmentation of the Cry1Ab protein was recently described (Lutz et al., 2005; Wiedemann et al., 2006) and might be due to feed processing, storage and enzymatic proteolysis in the ruminal GIT. The fragmentation also leads to an overestimation of the Cry1Ab protein concentration by means of ELISA.

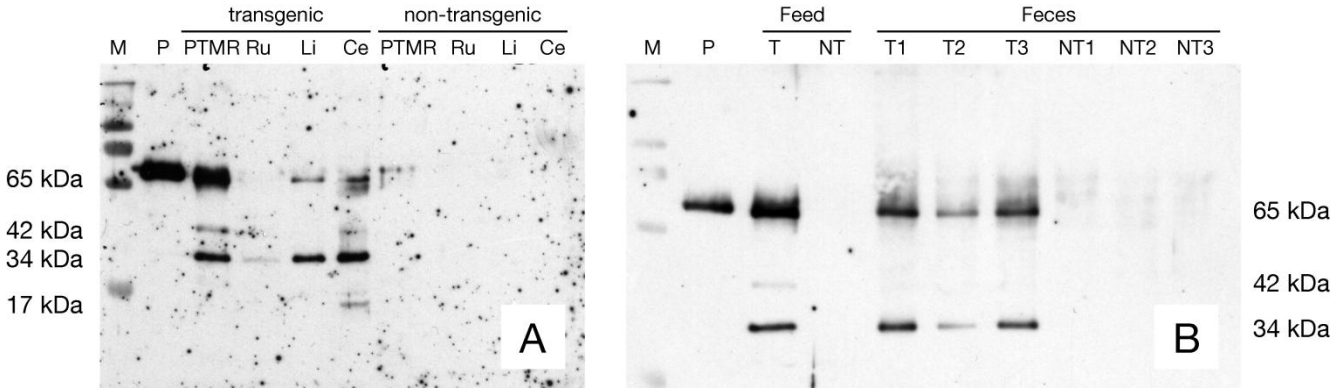

Figure 18: A) Immunoblot showing Cry1Ab protein fragments in GIT digesta (Ru, rumen; Li, large intestine; Ce, cecum) extracts from cows fed on transgenic and non-transgenic diets (PTMR). Each extract except rumen content contained 100 pg Cry1Ab protein (as measured in ELISA), whereas due to low concentration of Cry1Ab protein in rumen content extract 70 pg Cry1Ab protein was used. In non-transgenic feed and digesta extract respective amount of total protein was used. **B)** immunoblot showing Cry1Ab protein fragments in total protein extracts (60 μg) of transgenic (T) and non-transgenic feed (NT) and respective feces of transgenic (T1, T2, T3) and non-transgenic (NT1, NT2, NT3) fed dairy cows
Trypsin treated and HPLC purified Cry1Ab protein (100 pg) was used in both immunoblots as a positive control (P); in each blot, a marker (M) was used to estimate the fragment sizes

Conclusions

This is the first long-term experiment, investigating the metabolism of novel DNA and the Cry1Ab protein during feeding gm maize to lactating dairy cows for 25 months. Only short-term studies had been accomplished so far. To ensure a sensitive and specific detection of Cry1Ab protein and *cry1Ab* DNA fragments in samples of this long-term study, new quantification and detection methods were developed, optimized and validated according to existing guidelines of GMO testing. In previous studies, skim milk had been commonly used for recombinant DNA analysis. Hence, an extraction and detection method for DNA in whole milk was established and validated. Further, this long-term study was conducted using non-transgenic and transgenic maize components with equivalent feed values to assure a comparable feeding regime of the target and the control group.

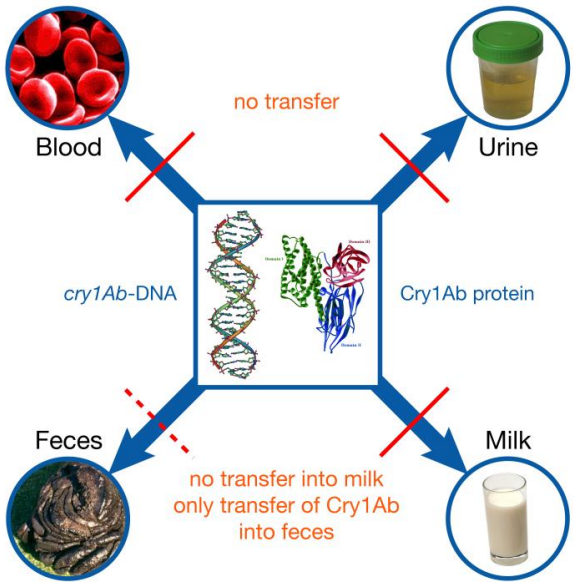

Figure 19: Schematic scheme of the results of this study concerning the potential transfer of novel DNA and the Cry1Ab protein

Results of the long-term study revealed a rapid degradation of DNA and protein due to the ensiling process whereas both components remained intact in maize kernels and cobs resulting in a daily intake of at least 6 mg Cry1Ab protein per cow. During digestion in the dairy cow *cry1Ab* DNA was rapidly metabolized. Data obtained by immunoblot analysis showed a degradation of the Cry1Ab protein via smaller immunoactive fragments of 42, 34 and 17 kDa. However, the degree of biological activity of these fragments needs to be studied. The degradation into immunoreactive fragments results in an estimation of total Cry1Ab protein including fragments using the ELISA technique. Nevertheless, the metabolism of the Cry1Ab protein is characterized by faster degradation processes in the bovine gastrointestinal tract in comparison to other proteins.

Neither the novel protein, nor the *cry1Ab* DNA was detected in whole milk within the LOD, considering milk from cows fed gm maize not different to milk from cows fed non-gm maize in any of the tests applied. After feeding gm maize for 25 months, an accumulation of transgenic components or a transfer of *cry1Ab* DNA or immunoactive fragments of the Cry1Ab protein into the body of the dairy cow could not be demonstrated (Figure 19).

On this account, this long-term study is an important step to face safety concerns regarding the use of gm maize (MON810) for animal and human consumption.

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Appendix

Appendix 1: Guertler P., Paul V., Albrecht C. and Meyer H. H.D. (2009) Sensitive and highly specific quantitative real-time PCR and ELISA for recording a potential transfer of novel DNA and Cry1Ab protein from feed into bovine milk. Anal. Bioanal. Chem. 393: 1629-1638

Appendix 2: Guertler P., Paul V., Steinke K., Wiedemann S., Preißinger W., Albrecht C., Spiekers H., Schwarz F.J. and Meyer H. H.D. (2009) Long-term feeding of genetically modified maize (MON810) – fate of *cry1Ab* DNA and novel protein during the metabolism of the dairy cow. J. Anim. Sci. submitted (July 13th, 2009)

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Appendix 1

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ORIGINAL PAPER

Sensitive and highly specific quantitative real-time PCR and ELISA for recording a potential transfer of novel DNA and Cry1Ab protein from feed into bovine milk

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Abstract To address food safety concerns of the public regarding the potential transfer of recombinant DNA (*cry1Ab*) and protein (Cry1Ab) into the milk of cows fed genetically modified maize (MON810), a highly specific and sensitive quantitative real-time PCR (qPCR) and an ELISA were developed for monitoring suspicious presence of novel DNA and Cry1Ab protein in bovine milk. The developed assays were validated according to the assay validation criteria specified in the European Commission Decision 2002/657/EC. The detection limit and detection capability of the qPCR and ELISA were 100 copies of *cry1Ab* μL^{-1} milk and 0.4 ng mL^{-1} Cry1Ab, respectively. Recovery rates of 84.9% (DNA) and 97% (protein) and low (<15%) imprecision revealed the reliable and accurate estimations. A specific qPCR amplification and use of a specific antibody in ELISA ascertained the high specificity of the assays. Using these assays for 90 milk samples collected from cows fed either transgenic (n=8) or non-transgenic (n=7) rations for 6 months, neither *cry1Ab* nor Cry1Ab protein were detected in any analyzed sample at the assay detection limits.

Keywords Bovine milk · ELISA · MON810 · Quantitative real-time PCR · Validation

Patrick Guertler and Vijay Paul have contributed equally to this study.

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Introduction

Genetically modified (GM) maize (Bt-maize; event MON810) is one of the most important new generation transgenic insect-resistant hybrid plant that has been genetically altered by insertion of the *cry1Ab* gene from a naturally occurring soil bacterium *Bacillus thuringiensis* (Bt), encoding Cry1Ab protein [1] to gain resistance against its major insect pest, the European Corn Borer (*Ostrinia nubilalis*). Since the past 12 years, after the first commercialized release and approval of GM crops in 1996, the global cultivation area of Bt-maize has increased along with other major biotech crops (soybean, cotton, and canola) reaching a total of 114.3 million ha in 2007 [2]. As a result, there is an increase in the availability of Bt-maize for human and livestock consumption. Despite this consecutive increase in global adoption of GM crops, there is an ongoing debate and increasing public concern about potential effects and the fate of recombinant DNA and protein in the food derived from animals fed GM crops.

Therefore, as a food safety authenticity measure, several countries worldwide including the European Union (EU) have implemented mandatory labeling for foods derived from the GM plants. In the EU, a tolerance limit of 0.9% has been set before mandatory GMO labeling in food/or feed ingredients [3]. Consequently, for GMO detection, identification, tracing and quantification, the analytical methodologies focused on two targets: the transgenic DNA or the novel protein expressed in a genetically modified organism. The most accepted techniques for accurate and specific detection of recombinant DNA and protein are the polymerase chain reaction (PCR) and the enzyme linked immunosorbent assay (ELISA). Various analytical methods [4–8] have been developed and routinely used for the monitoring of GMOs in raw materials and processed foods.

However, the possible transfer of transgenic DNA and protein to animal-derived products like milk, intended for human consumption, needs to be fully addressed. Previously described analytical methods for the detection of *cry1Ab* DNA in milk [9–12] either amplified fragments of transgenic DNA using event specific gene primers in qualitative PCR or quantified transgenic DNA in quantitative real-time PCR (qPCR) based on total plant DNA mass calibrations. Therefore, the quantification of recombinant DNA fragments using qPCR based on the copy number of inserted transgene calibration could give better details of even minute amounts of the transgenic material in the samples. Furthermore, these methods need proper validation before application in complex matrices like milk.

On the other hand, so far only one commercially available enzyme immunoassay kit [13] has been used for monitoring the novel Cry1Ab protein in milk. However, the used commercial kit designed for the GMO detection in plant materials has not been adequately validated. According to EU Regulation (EC) 882/2004, analytical methods used for food and feed control purposes must be validated before their use in control laboratories. Though, a validated quantitative method for the detection of event MON810 has been published by the Community Reference Laboratory for GM Food and Feed (http://gmo-crl.jrc.ec.europa.eu/summaries/Mon810_validation_report.pdf). However, to the best of our knowledge, there is no validated method for the quantification of recombinant DNA and protein from event MON810 by means of quantitative real-time PCR and ELISA in bovine milk.

Therefore, the aim of the present study was to optimize and validate a sensitive and specific extraction and detection method for recombinant DNA and the Cry1Ab protein in bovine milk samples. The methods were further used for the monitoring of presence or absence of the suspected recombinant DNA and Cry1Ab protein in the milk of multiparous cows fed GM maize or non-GM maize supplemented rations.

Materials and methods

Milk samples and feeding experiment

Milk samples from cows fed on non-GM diet were taken from a bulk milk storage tank and used for assay (quantitative real-time PCR and ELISA) optimization and validation. In addition, 54 milk samples from different cows reared at three farms maintaining three different breeds (Veitshof: 12 Brown Swiss cows, Grub: seven Bavarian Fleckvieh cows and, Hirschau: 35 Red Holstein cows) were collected in sterile Falcon tubes (50 mL) for ELISA validation.

To investigate the possible transfer and existence of novel DNA and Cry1Ab protein in the milk of cows fed on a ration supplemented with the GM maize (MON810), a 6-month feeding trial was conducted on 15 multiparous (2nd lactation) lactating Bavarian Fleckvieh cows. All cows were housed at the Bavarian State Research Center (LfL, Grub, Germany) and separated into a target group (n=8) fed on a ration containing GM maize (MON810) and a control group (n=7) fed conventional maize. Daily diet of cows contained a partial mixed ration. The feed composition is shown in Table 1. According to the milk yield, further concentrates (40.4% maize kernels, 34.4% rapeseed meal, 19.9% molasses dried beet pulp, 3.2% mineral mixture and 2.4% urea) were offered above 22kg milk yield per day. Milk samples were taken monthly during the morning milking and stored at −20 °C until analyzed.

For Cry1Ab protein quantification and assay validation in ELISA, the skim milk was prepared by centrifugation of whole milk (fresh whole milk, pooled tank milk, and thawed frozen milk samples) at 3,000×g for 15 min at 4 °C in inversely (lid down) placed centrifuge tubes. After centrifugation, the skim milk was collected in clean vials by decantation and further used in the ELISA.

DNA analysis

To optimize and validate a sensitive DNA extraction method from milk, whole milk samples were spiked with the genomic DNA isolated from transgenic maize (MON810) containing different copy numbers of *cry1Ab*. Therefore, genomic DNA was first isolated from MON810 using an optimized CTAB-based protocol published by the Federal Office of Public Health (FOPH, Berne) [14].

Genomic DNA extraction from maize

Three hundred milligrams of grounded GM maize (MON810) kernels and 800 μL of a CTAB-extraction buffer

Table 1 Feed composition of the daily animal diet

Component	Ratio (DM)
Maize silage[a]	41.9%
Maize cobs[a]	21.2%
Grass silage	11.0%
Straw	5.9%
Molasses	1.4%
Concentrates	18.6%
Rapeseed meal	51.1%
Maize kernels[a]	41.2%
Mineral mixture	5.3%
Urea	2.4%

[a] Ingredients contained 100% MON810 in GM ration and conventional maize in non-GM ration

were added to FastPrep-tubes containing 300-mg beads (Lysing Matrix D, MP Biomedicals, Germany). The mixture was homogenized (two times for 30 s at a speed of 5.5 m s^{-1}) using the FastPrep FP120 (MP Biomedicals, Germany) and cooled on ice between the runs, followed by Proteinase K (Roth, Germany) digestion. Samples were incubated overnight at 60 °C and subsequently centrifuged (at 16,000×*g* for 15 min at 4 °C). The supernatant was transferred into a new tube, RNAse A (Roth, Germany) was added and the mixture was incubated for 15 min at 60 °C. After centrifugation (1 min at 16,000×*g* and room temperature), the clear supernatant was transferred to a new tube and 600 µL chloroform was added. The tubes were mixed immediately and centrifuged for 10 min at 16,000×*g* and 4 °C. The upper watery phase, containing the DNA, was collected and added to a tube prefilled with 500 µL 2-propanol (80%). Glycogen (Sigma, Germany) was added into the lid and the tubes were inverted four times, followed by an incubation step of 60 min at room temperature (RT). The DNA was pelleted by centrifugation (10 min at 16,000×*g* and 4 °C) and washed with 500 µL ethanol (70%). Wizard SV Columns (Promega, Germany) were placed into collection tubes and loaded with the samples, followed by a centrifugation step of 11,000×*g* and 4 °C for 2 min. The columns were washed twice using 700 µL 2-propanol (80%), placed into new tubes and dried for 5 min at RT. Finally, the DNA was eluted using 50 µL of a pre-warmed (70 °C) Tris–HCl buffer and stored at −20 °C until used.

DNA extraction from whole milk

For the assay validation and standard preparation for quantitative real-time PCR, 300 µL of whole milk was spiked with the extracted genomic DNA containing different copy numbers of the *cry1Ab* (10 to 10^6 copies). Copy numbers were calculated following the formula: (genomic DNA concentration in pg µL $^{-1} \times 6.0233 \times 10^{23}$ copies mol^{-1})/(haploid maize genome size (bp)$\times 660 \times 10^{12}$) [15]. Therefore, a genome size of 2.5 Mbps for the haploid maize genome [16] was used for the conversion of DNA concentration (pg μL^{-1}) into copy numbers. Hence, a mass of 2.74 pg of genomic DNA from transgenic maize contained one copy of *cry1Ab* DNA.

A previously published guanidinhydrochloride-based extraction protocol [14] was optimized to re-isolate the recombinant DNA from the spiked milk samples. In brief, 860 µL extraction buffer, 100 µL guanidinhydrochloride solution, and 40 µL Proteinase K (20 mg mL^{-1}) were added to the spiked milk samples. The mixture was incubated at 60 °C overnight while shaking and subsequently centrifuged for 10 min at 12,000×*g*. The clear supernatant was transferred into a new 1.5 mL tube and RNA was digested by RNAse A exposure (5 min at 60 °C while shaking). Wizard SV Columns were placed into collection tubes and loaded with the samples, followed by a centrifugation step of 10,000×*g* and 4 °C for 2 min. The column was washed twice using 800 µL 2-propanol (80%). Remaining 2-propanol was removed by drying the column for 10 min. Finally, DNA was eluted with 50 µL pre-warmed (70 °C) elution buffer and stored at −20 °C until analyzed.

For subsequent analysis of milk samples collected from the feeding trial, genomic DNA was extracted from 300 µL of raw milk sample. Concentrations of DNA in the extracted samples were determined by photometrical analysis using a Biophotometer (Eppendorf, Germany). The purity of DNA was evaluated by using UV absorption ratios of 260/280 nm and 260/230 nm.

Conventional PCR

Qualitative endpoint PCR was performed using 100 ng of genomic DNA (isolated from *cry1Ab* spiked milk) to amplify a 354 bp fragment of *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*, using forward primer 5′-ATCACTGCCACCCAGAAGAC-3′, reverse primer 5′-CCCAGCATCGAAGGTAGAAG-3′) as a positive DNA extraction control for endogenous reference gene and a 206 bp fragment of the *cry1Ab* gene [17] to verify the extraction method. The PCR mix consisted of 1× GoTaq Green Master Mix (Promega, Germany), 0.8 µM forward and reverse primers (Metabion, Germany). PCR-grade water was added resulting in a final volume of 25 µL. Water served as a negative control, while genomic DNA (100 ng) from GM maize served as a positive control for *cry1Ab* amplification. The following cycling conditions were used: initial denaturation at 94 °C for 5 min, denaturation at 94 °C for 30 s, annealing at 55 °C (*GAPDH*) or 60 °C (*cry1Ab*) for 30 s, extension at 72 °C for 30 s and final extension at 72 °C for 5 min. A total of 35 cycles was completed for *GAPDH*, 40 cycles for *cry1Ab*, respectively. The PCR product was separated by gel electrophoresis and visualized by staining with GelRed (Biotium, Hayward, USA).

Quantitative real-time PCR (qPCR)

Quantification of a 206 bp fragment of the *cry1Ab* in milk was carried out using the LightCycler-system (Roche Diagnostics, Germany) with 1 µL DNA template (standard or sample genomic DNA extracted from 300 µL milk), 1 µL LC FastStart DNA Master SYBR Green I (Roche Diagnostics, Mannheim, Germany), 4 pM of each primer and 3 mM $MgCl_2$ under the following cycling conditions: initial denaturation at 95 °C for 10 min to activate the DNA polymerase and to ensure complete denaturation of the DNA samples, denaturation at 95 °C for 15 s, annealing at 60 °C for 10 s and extension for 25 s. A fourth step at 86 °C was

added to remove unspecific signals before fluorescence acquisition. The PCR was performed in a reaction volume of 10 μL and was composed of 40 cycles. All runs included a negative control consisting of PCR-grade water. Additionally, a melting curve analysis was accomplished to check for correct amplification by melting temperature of the expected product. DNA products were sent for commercial sequencing to verify the specificity of the PCR product (data not shown). Samples were measured in duplicates and analyzed using the standard curve method. A standard curve was created by using 1 μL of re-isolated genomic DNA after spiking 300 μL of milk samples with different copy numbers of *cry1Ab* (10 to10^6), following the extraction procedure as described above. Therefore, genomic DNA of MON810 maize containing 10, 10^2, 10^3, 5×10^3, 10^4, 5×10^4, 10^5, 5×10^5 and 10^6 copies of *cry1Ab* per 300 μL of whole milk were re-isolated and dissolved in 50 μL elution buffer. Further, 1 μL of each standard concentration was used to generate the standard curve.

Data obtained by real-time PCR was analyzed using the LightCycler-system software (Roche, Germany). Data expression levels were recorded as the cycle threshold (CT) value, which was derived using the Second Derivative Maximum Method, identifying the CT of a sample as the cycle number where the sample's fluorescence is detected above the background and the amplification is in the exponential phase.

The dynamic range and the PCR efficiency were determined by plotting the CT values against the log of the estimated DNA copy number of the calibrators to generate a standard curve. The slope of the standard curve was used for the amplification efficiency calculation using the following formula: $[(10^{1/\text{slope}})-1]\times100$.

Assay validation

To specify the efficiency of the optimized extraction method, the recovery rate was determined by re-isolation and quantification of *cry1Ab* in milk samples spiked with three different copy numbers (5,000, 50,000 and 500,000 copies; six replicates each). Furthermore, inter- and intra-assay coefficients of variation (CV) were verified by analysis of three standard samples within the standard curve.

Protein analysis

Reagents

All the reagents were of analytical grade and supplied by Merck (Darmstadt, Germany) unless specified otherwise. HPLC-purified trypsin-activated Cry1Ab protein for standard preparation was generously provided by Dr. William J. Moar, Auburn University, USA.

Immunoaffinity purified Cry1Ab protein specific polyclonal antibody was raised in rabbits and labeled with biotin as described in detail elsewhere [18].

Antibody coating buffer (CB) was 50 mM sodium carbonate/bicarbonate buffer pH 9.6. Assay buffer (PBST) was phosphate-buffered saline (PBS; 8 mM sodium phosphate, 137 mM NaCl, 2.7 mM KCl, 1.5 mM potassium phosphate pH 7.4) containing 0.1% Tween 20.

Matrix-matched calibrators (0.1 ng mL^{-1} to 25 ng mL^{-1}) and controls (0.2, 2.0 and 8.5 ng mL^{-1}) were prepared by fortifying Cry1Ab protein in analyte free pooled tank skim milk collected from cows fed rations containing non-transgenic maize.

ELISA procedure

The assay was performed in 96-well microtiter plates (Maxisorp™, Nunc, Denmark) coated with the immunoaffinity purified anti-Cry1Ab protein rabbit polyclonal antibody (capture antibody) at a concentration of 0.02 μg well^{-1} in 100 μL coating buffer. Then, the plates were incubated overnight at 4 °C under gentle shaking. After 12 h, the contents of the coated plates were decanted and remaining unbound active sites on each well were blocked by incubating 300 μL of 1% bovine serum albumin (BSA; SERVA, Heidelberg, Germany) in PBST well^{-1} for 1 h at RT while shaking. After decantation of the blocking buffer, the capture antibody coated and blocked plates were stored frozen at −20 °C until used. At the time of assay, frozen plates were thawed to RT and washed twice with assay buffer using a 96-well microplate washer (SLT Lab Instruments, Tecan, Germany). Aliquots of 40 μL matrix-matched calibrators (0.1 ng mL^{-1} to 25 ng mL^{-1}), controls (0.2, 2.0 and 8.5 ng mL^{-1}) and unknown skim milk samples were added to respective wells of microtiter plate (in duplicates) followed by the addition of 60 μL assay buffer. Plates were incubated for 3 h at RT while shaking, and washed four times with assay buffer. Then, 100 μL biotinylated detection antibody (10 ng mL^{-1} diluted in assay buffer) was added to each well, and incubated at RT for 1 h on a plate shaker. After four washing steps with assay buffer, streptavidin–horseradish peroxidase enzyme conjugate (Roche Diagnostics, Mannheim, Germany; 100 μL, diluted 1:15,000 times in assay buffer) was added to each well and incubated for 15 min at room temperature. After four washes, 150 μL 3,3′,5,5′-tetramethylbenzidine (TMB; Sigma, Germany) enzyme substrate solution was added to each well and plates were incubated for 40 min at room temperature in dark. Thereafter, the enzymatic reaction was stopped by addition of 2 M sulfuric acid (50 μL well^{-1}) and the absorbance was then read at 450 nm in a microplate reader (Sunrise, Tecan, Germany). The calibration curve for Cry1Ab protein was constructed using online Magellan V6.1 software (Tecan,

Austria) and the concentration of Cry1Ab protein in unknown samples was determined by interpolation. All data are presented as ng of Cry1Ab protein mL^{-1} milk.

Optimization of ELISA

Several parameters of assay development, including the optimal choice of assay buffer and the specificity of immunoaffinity purified polyclonal Cry1Ab protein antibody, were previously optimized [18]. However, the basic sandwich enzyme immunoassay protocol is used with variation in a series of assays to select the optimal concentrations of the anti-Cry1Ab capture and biotin-labeled detection antibodies, and to choose an appropriate sample volume of skim milk for the assay. A sensitive matrix-matched calibration curve was generated to reduce the biasness in analysis results due to the matrix interference.

Assay validation

The assay was validated according to the criteria specified in the adopted European Commission Decision 2002/657/EC [19] for the performance and validation of screening and confirmatory analytical methods.

Decision limit (CCα) and detection capability (CCβ) ELISA validation was carried out using 54 different milk samples (blanks), known to be free of Cry1Ab protein, collected from cows fed on non-transgenic ration reared at three different farms. The samples were analyzed using a sandwich ELISA as described above to demonstrate the range of blank matrix effects and to determine CCα and CCβ. CCα is equal to the average background noise plus three times the signal to noise level recorded for the Cry1Ab protein in the blanks. CCβ was calculated by using the equation $CC\beta = CC\alpha + 1.64 \times SD_S$. SD_S is the standard deviation obtained for above 54 blanks fortified at the spike concentration level of CCα. Calculation for α- and β-error were carried out from the Cry1Ab protein background noise level in 54 blanks and fortifying the same samples at concentration level of CCβ value. α-error is the percentage of blank values exceeding the CCα value. β-error is represented by the percentage of blank samples showing signals below the CCα value when fortified at the concentration level of CCβ.

Recovery and precision Recovery and precision were determined in accordance with Commission Decision 2002/657/EC by spiking blanks (pooled tank milk) with Cry1Ab protein. Recovery was calculated for 6 aliquots of blank skim milk per spike concentration level for five different concentration levels (0.6, 0.8, 1, 10, and 20 ng mL^{-1}). Precision was expressed by inter- and intra-assay CV and calculated from the analysis of blank skim milk aliquots fortified with Cry1Ab protein at three (controls) different concentration levels of 0.2, 2.0, and 8.5 ng mL^{-1} (three determinants per assay) in 11 assays performed on different days.

Application of validated methods in milk sample analysis of feeding trial

Optimized and validated qPCR and ELISA methods were used for the analysis of milk samples collected from the feeding study to monitor the suspicious presence of recombinant DNA and Cry1Ab protein in milk.

Results and discussion

Quantification of recombinant DNA

Genomic DNA from maize (event MON810) was extracted containing the *cry1Ab* gene in order to spike whole milk samples with different copy numbers of this gene. Genomic DNA extraction from maize kernels resulted in high concentrations up to 900 ng μL^{-1} with a high DNA quality indicated by 260/280 nm ratio values of 1.6–1.9.

Whole milk was spiked with predefined copy numbers of *cry1Ab* ranging from 10 to 10^6 copies to assess an assay validation. Photometrical analysis of the isolated milk samples revealed genomic DNA concentrations ranging from 45 to 250 ng μL^{-1} and integrity values varying from 0.9 to 1.6. The specificity and sensitivity of the primer pair for the amplification of a 206-bp fragment of *cry1Ab* DNA from the extracted genomic DNA template from spiked milk sample (containing 10 to 10^6 copies) was tested in conventional PCR along with a negative control (unspiked milk) and a positive control (maize genomic DNA). Specific amplicons (206 bp) were reproducible seen with the genomic DNA extracted from spiked milk containing $\geq 10^3$ copies of *cry1Ab* and positive control (Fig. 1) Absence of the non-specific product and amplicon sequence analysis (data not shown) ensured the specificity of PCR in sample matrix (milk). To ensure the accuracy of genomic DNA extraction from milk, each extracted sample was further tested for the amplification of a 354-bp *GAPDH* fragment (extraction positive control). Amplification of a bovine *GAPDH* fragment (354 bp) by means of conventional PCR confirmed the successful DNA extraction from bovine milk (data not shown).

qPCR

Based on qPCR, 100 copies of *cry1Ab* per μL were reproducibly detectable by amplification of a 206-bp fragment. Hence, the limit of quantification was set 100

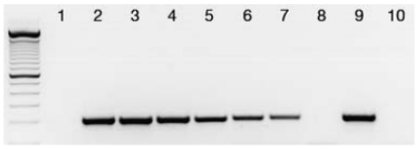

Fig. 1 Specific amplification of a 206-bp fragment of *cry1Ab* in spiked milk samples (10 to 10^6 copies) by means of conventional PCR. 10 and 10^2 copies of *cry1Ab* per 300 µL milk were not reproducibly detectable, therefore, the results are not shown in this figure. *Lane 1* Non-spiked milk sample, *lane 2* milk sample spiked with 10^6 copies of *cry1Ab*, *lane 3* milk sample spiked with 5×10^5 copies of *cry1Ab*, *lane 4* milk sample spiked with 10^5 copies of *cry1Ab*, *lane 5* milk sample spiked with 5×10^4 copies of *cry1Ab*, *lane 6* milk sample spiked with 10^4 copies of *cry1Ab*, *lane 7* milk sample spiked with 5×10^3 copies of *cry1Ab*; lane 8: milk sample spiked with 10^3 copies of *cry1Ab*, lane 9: positive control (genomic DNA extracted from GM maize), *lane 10* negative control (water)

copies/µL genomic DNA. The qPCR was able to detect recombinant DNA concentrations in a dynamic range of 10^2 to 10^5 copies/µL with an average amplification efficiency of 98% and an average R^2 coefficient of 0.98 (Fig. 2). The values of the amplification efficiency and the values of the R^2 coefficient were within the range of the Minimum Performance Requirements for Analytical Methods of GMO Testing published by the European Network of GMO Laboratories (ENGL).

A melting curve analysis of different standard concentrations (10^2 to 10^5 copies of *cry1Ab*) showed a specific amplification by melting temperature (Tm) 89 °C of the specific product (Fig. 3). The specificity of our qPCR assay was further ensured by subsequent amplicon sequence analysis (data not shown). A mean recovery rate of 84.9% (n=3, six replicates each), an intra-assay CV of 0.15 (n=9) and an inter-assay CV of 0.78 (n=9, three replicates each)

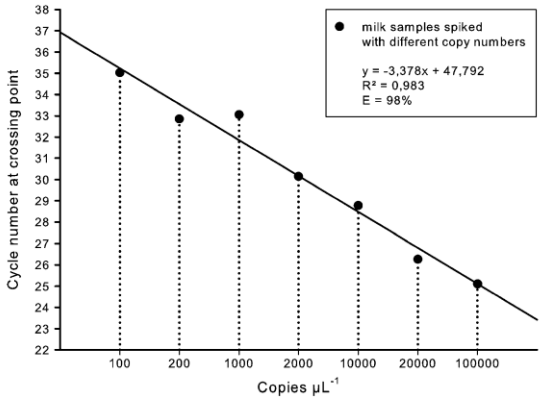

Fig. 2 An exemplary standard curve of milk samples spiked with different copy numbers of *cry1Ab* as used in the qPCR. The standard curve shows the linearity between 100 and 100,000 copies μL^{-1}

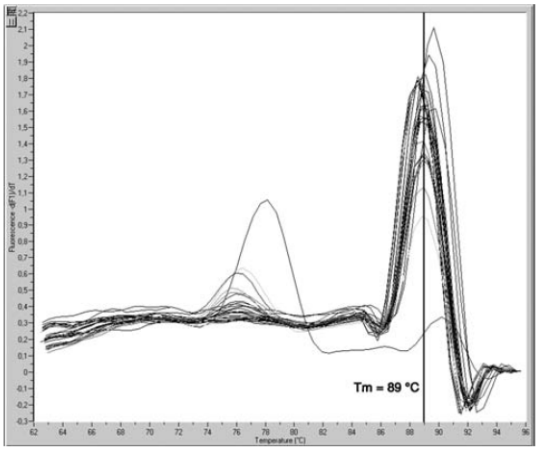

Fig. 3 A melting curve analysis of different standard concentrations (10^2 to 10^5 copies of *cry1Ab*) showing a specific amplification by melting temperature (Tm) 89 °C of the specific product (a 206 bp fragment of *cry1Ab*)

illustrate the suitability of the extraction and quantification procedure for novel DNA in whole milk (Table 2).

Quantification of the Cry1Ab protein

Optimization of assay

The optimal concentrations of the anti-Cry1Ab capture and biotin-labeled detection antibodies were determined by a two-dimensional checkerboard titer test against the fixed concentration of 1 ng per well Cry1Ab protein. The titer test was performed on a 96-well microtiter plate by coating wells with a dilution range of 0.008 µg to 0.1 µg per well of capture antibody and measuring the absorbance against the fixed concentration of 1 ng $well^{-1}$ Cry1Ab protein using a dilution range of 0.063 to 2 ng/well of detection antibody. The ELISA values (absorbance at 450 nm) obtained after substrate reaction were used for choosing the optimal concentration of antibody pairs. The concentrations of the antibody pair was chosen, when the maximum absorbance values (A_{max}) were around 1.0 to 1.5 and the respective values of negative antiserum (blanks) was lower than 0.1. As a result, the optimal concentrations of antigen capture and biotin-labeled detection antibodies were 0.2 µg mL^{-1} and 10 ng mL^{-1}, respectively. The optimized antibody concentrations for pairing Cry1Ab protein were used in subsequent assays.

Matrix interference and assay sensitivity

One of the common challenges of immunoassays is the matrix interference. This can be reduced by either sample dilution with buffers or by using a matrix-matched calibra-

Table 2 Analytical precision for real-time quantitative PCR and ELISA in spiked bovine milk

Coefficient of variation (CV)[a]	Real-time qPCR (DNA)				ELISA (Protein)			
	Spiked *cry1Ab* controls			Mean	Spiked Cry1Ab controls			Mean
	C1 10^4 copies μL^{-1}	C2 10^3 copies μL^{-1}	C3 10^2 copies μL^{-1}	CV	C1 0.2ng mL^{-1}	C2 2ng mL^{-1}	C3 8.5ng mL^{-1}	CV
Intra-assay (%)	0.03	0.07	0.36	0.15	12.3	6.7	8.2	9.1
Inter-assay (%)	0.15	0.08	2.11	0.78	13.7	7.4	8.0	9.7

Milk samples collected from cows fed rations containing non-transgenic maize

[a] Coefficients of variation at different spike concentrations of *cry1Ab* DNA and Cry1Ab protein (three determinants per assay) in total 11 independent assays.

tion curve. Here, we have studied the influence of the matrix (analyte free tank skim milk) volume on the assay sensitivity by using Cry1Ab protein calibration curves in assay buffer, compared with the addition of different volumes of skim milk (10, 20, and 40 μL). The results indicated that the addition of analyte free skim milk has inhibited the A_{max} and affected the sensitivity of the calibration curve. However, no change was recorded in the absorbance values (A_{max}) with the increased sample size from 10 to 40 μL per well. As a result, a matrix-matched calibration curve using 40 μL sample size (skim milk volume) was used in subsequent assays to nullify the biasness in analysis due to probable matrix interferences (Fig. 4).

A typical matrix-matched Cry1Ab protein calibration curve using optimized ELISA conditions is shown in Fig. 4. The developed sandwich assay allowed the determination of Cry1Ab protein over the dynamic range (<20% CV between the replicates of calibrators) from 0.1 to 25 ng mL^{-1} with an analytical limit of 0.1 ng mL^{-1}.

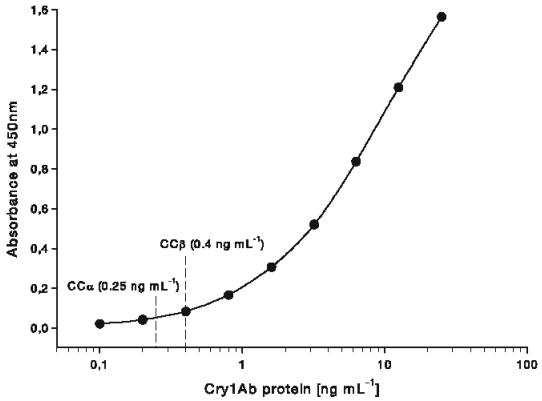

Fig. 4 A typical matrix-matched calibration curve for Cry1Ab protein in bovine skim milk

Milk samples and assay validation

Decision limits (CCα) and detection capability (CCβ) Analysis of 54 blank skim milk samples by ELISA showed the background noise for Cry1Ab protein ranging from 0.1–0.32 ng mL^{-1} (mean 0.11 ng mL^{-1}; Fig. 5). The decision limit (CCα) calculated from the mean background noise (0.11 ng mL^{-1}) plus three times signal to noise level (SD; 0.046 ng mL^{-1}) was 0.25 ng mL^{-1}. When same 54 blanks were fortified with Cry1Ab protein at the concentration level of CCα (0.25 ng mL^{-1}), the values ranged from 0.17–0.73 ng mL^{-1} (mean; 0.22 ng mL^{-1}; SD_S 0.08 ng mL^{-1}; Fig. 5). The detection capability (CCβ) calculated from the equation $CC\beta = CC\alpha + 1.64 \times SD_S$ was 0.4 ng mL^{-1}. The observed Cry1Ab protein values for the blanks fortified at concentration level of CCβ (0.4 ng mL^{-1}) ranged from 0.28–0.71 ng mL^{-1} (mean 0.42 ng mL^{-1}) (Fig. 5). Detection capability (0.4 ng mL^{-1}) and threshold value of 0.28 ng mL^{-1} (the lowest observed 0.4 ng mL^{-1} fortified sample) laid the basis for selection of the samples for confirmatory analysis. Therefore, the samples with a concentration level at and above 0.28 ng mL^{-1} must be analyzed by any other confirmatory method to draw a final conclusion. The assay β-error is zero since no false negative (false compliant) results were obtained for 0.4 ng mL^{-1} fortified blank skim milk samples. This satisfies EU Commission Decision 2002/657/EC [19] which states that screening assays must "have a false compliant rate of <5% (β-error) at the level of interest". Similarly, the α-error (false non-compliant) is <5% as one blank value exceeded the CCα (0.25 ng mL^{-1}) value.

Recovery and precision The analytical performance of the developed enzyme immunoassay was assessed by spiking matrix samples (whole tank milk) with the Cry1Ab protein. The immunoassay performed well when it was applied to spiked whole milk samples and recoveries in skim milk ranged from 88 to 104% (mean value of 97%; Table 3). The Cry1Ab protein fortified skim milk blanks at spike

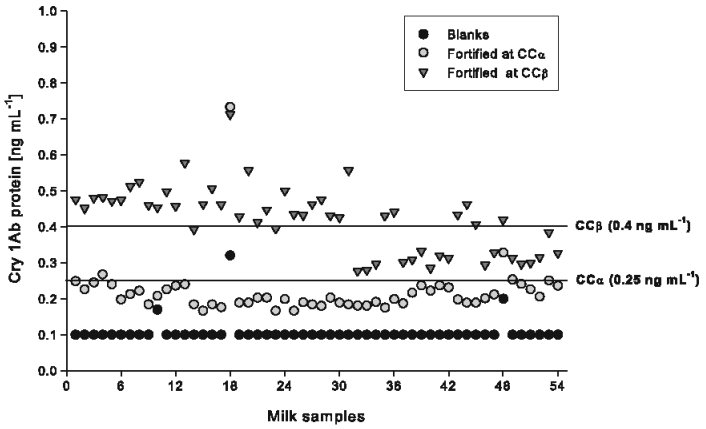

Fig. 5 Determination of decision limit (CCα) and detection capability (CCβ) and α- and β-errors for the Cry1Ab protein ELISA in bovine skim milk. CCα calculated as mean background noise plus three times S/N ratio from the 54 blanks (*filled circle* skim milk collected from 54 cows fed non-transgenic ration). Detection capability was calculated from the Cry1Ab protein fortified blanks (*empty circle* $n=54$) at concentration of 0.25 ng mL^{-1} (CCα). Percentage of blanks showing the signals above the CCα value (0.25 ng mL^{-1}) indicated the <5% α-error. Zero β-error was indicated by the spiked blanks (*inverted filled triangle*) fortified at CCβ value of 0.4 ng mL^{-1}

concentrations of 0.2, 2.0, and 8.5 ng mL^{-1} (three determinants per assay) in 11 independent assays showed a good assay precision, with intra- and inter-assay CVs of 9.1 and 9.7%, respectively (Table 2).

The analytical performance of the assay indicates that it can be used for monitoring concentration levels of Cry1Ab protein in bovine milk.

Surveillance of recombinant DNA and Cry1Ab protein in milk of cows fed transgenic maize (MON810)

In total, 90 milk samples collected from eight transgenic and seven non-transgenic ration-fed cows during 6 months feeding trial were analyzed for the suspicious presence or absence of the *cry1Ab* and Cry1Ab protein using developed qPCR and ELISA, respectively. In both feeding groups, no milk sample was found suspicious for the presence of novel DNA and protein (Fig. 6) at assay detection limits. These results are in accordance with the few previous findings that also reported the absence of recombinant DNA and protein in milk [10, 13, 20–22]. Contrary to this, Agodi et al. [9] detected small fragments of recombinant DNA in analyzed milk samples from the Italian market, which they suspected was due to feed and fecal contamination during milking of cows offered GM diets. Degradation of the intact transgenic DNA and protein during feed processing, storage, and ruminal plus intestinal digestion might be the reasons for the absence of these recombinant fragments in milk. Previous studies on GM maize [23–25] reported that the degradation of plant DNA starts directly after harvest due to bacterial activity and is also greatly influenced by feed

Table 3 Recoveries achieved for *cry1Ab* DNA and Cry1Ab protein determination in spiked bovine milk

cry1Ab DNA			Cry1Ab protein		
Amount added [copies 300 μL^{-1}]	Amount measured	Recovery [%]	Amount added [ng mL^{-1} milk]	Amount measured	Recovery [%]
5×10^5	$4.66\times10^5\pm1\times10^5$	93.1	0.6	0.625±0.026	104.1
5×10^4	$5.47\times10^4\pm1.84\times10^4$	109.3	0.8	0.784±0.072	97.9
5×10^3	$2.61\times10^3\pm1.1\times10^3$	52.2	1.0	0.879±0.041	87.9
			10	9.333±0.617	93.3
			20	20.230±1.547	101.2
Mean recovery [%]		84.9	Mean recovery [%]		96.9

Milk samples collected from non-GM ration-fed cows.

Mean value±standard deviation (six replicates)

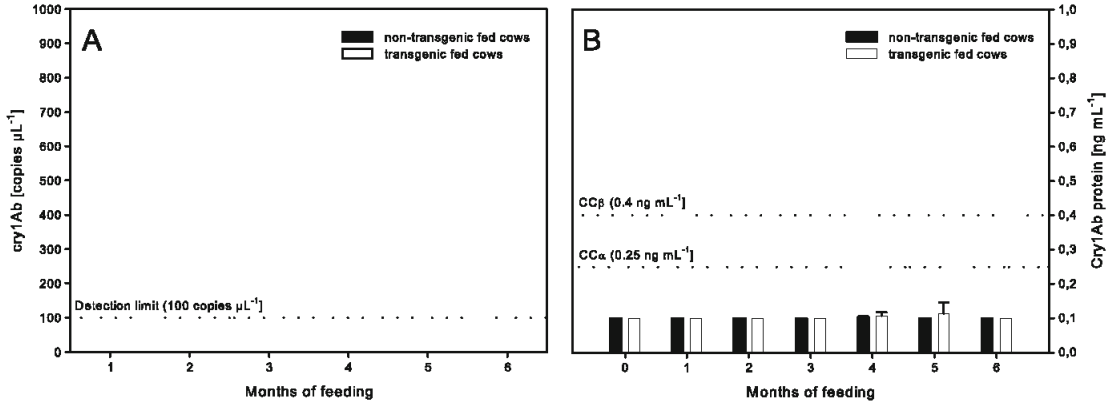

Fig. 6 Background signals for *cry1Ab* DNA and Cry1Ab protein in milk of cows fed transgenic (n=8) and non-transgenic ration (n=7). No sample found suspicious for the presence of *cry1Ab* DNA (**a**) and Cry1Ab protein (**b**) in milk. All the ELISA values were below the decision limit (0.25 ng mL^{-1}) and detection capability (0.4 ng mL^{-1}). The data is presented as mean (±SD) values

treatment. After feed intake, both the DNA and protein are further degraded within the bovine gastrointestinal tract (GIT) [26, 27]. So, it is very unlikely that intact DNA passes the GIT and is available for absorption via the Peyers' Patches [28]; though small fragments of non-transgenic plant DNA were found in mouse cells and tissues that are part of the immune system [29]. Also, small fragments of multicopy plant chloroplast DNA were detected in some animal tissues (blood, tissue, milk) [12, 22, 30, 31], but so far, no study revealed a transfer of novel DNA from the blood circle to the mammary gland in any species. Authors suggested to further investigate the mechanisms of molecule transport across the epithelial layer of the GIT into blood stream.

Another reason for the absence of Cry1Ab protein in milk could be the lack of the absorption mechanisms involved in the transfer of this protein from the gut into blood stream to enter the mammary gland for final secretion in milk. This could be further supported by the findings reporting the lack of Cry1Ab protein specific receptors on bovine intestinal epithelium [32, 33]; though so far, no single-copy genes (including transgenes) and Cry1Ab protein were detected in milk of cows fed GM ration. However, to ensure the potential absence or presence of transgenic DNA and protein in milk of cows, long-term GM-feeding effects need to be further evaluated. Therefore, the validated methods for the quantification of GM DNA and Cry1Ab protein will further facilitate the reliable analysis of milk samples.

To the best of our knowledge, these are the first available methods for a specific detection of *cry1Ab* DNA and the Cry1Ab protein in milk of cows fed transgenic maize fulfilling all the validation criteria as prescribed in the guidelines of EU-Decision 2002/657/EC. Further, these methods can be used for reliable monitoring of milk samples for unwanted suspicious presence of *cry1Ab* DNA and Cry1Ab protein to address the authenticity concerns of respective consumers.

Conclusions

Highly specific and sensitive quantitative real-time PCR and sandwich ELISA have been developed for the *cry1Ab* DNA and Cry1Ab protein determination at low levels of *cry1Ab* (100 copies) and Cry1Ab protein (CCβ 0.4 ng mL^{-1}) in bovine milk, respectively. The developed assays satisfy the performance and validation criteria laid down by Commission Decision 2002/657/EC. Both the methods performed well when applied to spiked milk samples and mean recoveries in milk were 84.9 and 96.9% for *cry1Ab* and Cry1Ab protein, respectively. Based on the good recovery and assay performance, the assays found suitable for recording a potential transfer of novel DNA and Cry1Ab protein into milk of cows fed a transgenic ration supplemented with MON810. When applied for the surveillance of novel DNA and immuno detective protein fragments from Bt-maize in milk of cows fed transgenic ration for a 6-month feeding study, no milk sample was suspicious for the presence of recombinant DNA and the Cry1Ab protein.

These validated methods could further be used for the analysis of milk samples collected from the cows fed continuously for a long-term on transgenic ration to monitor the unwanted suspicious potential existence of recombinant DNA and Cry1Ab protein in the milk, which could answer the questions raised on the long-term GM feeding and food authenticity concerns of respective consumers.

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Appendix 2

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Long-term feeding of genetically modified corn (MON810) – fate of *cry1Ab* DNA and novel protein during the metabolism of the dairy cow[1]

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ABSTRACT

Genetically modified maize (MON810) is altered by insertion of the *cry1Ab* gene into the plant genome encoding for the Cry1Ab protein to gain resistance against one of the major pests, the European Corn Borer (*Ostrinia nubilalis*). As genetically modified corn has been increasingly used for human and livestock consumption since 1996, public concern about potential effects of MON810 have been expressed. However, only short-term studies were conducted so far on the fate of recombinant DNA or the novel protein after feeding genetically modified plants to livestock. Therefore, in a long-term study of 25 months, 36 lactating dairy cows were fed on rations containing either genetically modified corn (MON810, N=18) or the non-transgenic variety (N=18) to investigate the fate of *cry1Ab* DNA and Cry1Ab protein during the metabolism of the dairy cow. Feed samples were taken weekly, while feces, blood and milk samples were taken monthly, urine samples were taken bimonthly. All samples were analyzed for the presence of *cry1Ab* DNA by means of conventional (feces, blood, urine) and quantitative real-time PCR (feed, milk). A sensitive and highly specific ELISA was newly developed to quantify immunoreactive fragments of the Cry1Ab protein. The assays were validated according to the European Commission Decision 2002/657/EC and the "Minimum Performance Requirements for Analytical Methods of GMO Testing" published by the European Network of GMO Laboratories. Non-transgenic feed samples were free of recombinant DNA or the novel protein within the limit of detection, while in transgenic feed samples both, a 206bp fragment of *cry1Ab* and immunoreactive fragments of the Cry1Ab protein were detected. All blood, milk and urine samples were free of recombinant DNA and novel protein. Feces samples were not found positive for *cry1Ab*, but immunoreactive fragments of the Cry1Ab protein were detected in all transgenic feces samples. The absence of *cry1Ab* and fragments of the Cry1Ab protein in milk of cows fed genetically modified corn classify milk not different from milk of cows fed non-transgenic corn in any of the tests applied.

KEYWORDS

dairy cow, ELISA, genetically modified corn, long-term feeding, MON810, quantitative real-time PCR

INTRODUCTION

Since the commercial release in 1996, genetically modified (GM) corn has been increasingly used for livestock production and human consumption. The global cultivation of GM corn reached 37.3 million hectares in 2008 (Clive, 2008). Herbicide resistance and insect resistance are the most common traits to be incorporated in GM corn. In Bt-corn (MON810), the insect resistance is accomplished by insertion of the *cry1Ab* gene from *Bacillus thuringiensis* (Whiteley and Schnepf, 1986) into the plants' genome. The translated Cry1Ab protein desintegrates midgut epithelial cells of target insects by forming pores in the cell membrane (Bravo et al., 2007; de Maagd et al., 2001), which leads to swelling of the cells resulting in osmotic cell lysis (English and Slatin, 1992). Due to the lack of receptors, Cry1Ab is considered as non-toxic for mammals (Shimada et al., 2006b). No effects on cell morphology, albumin secretion or LDH release were reported after Cry1Ab exposure to mammalian cells (Shimada et al., 2006a). Besides that, no effect on cell proliferation and ATP level was observed after long-term exposure of Cry1Ab protein to ruminal epithelial cells (Bondzio et al., 2008). Several short term studies on dairy cows elucidated that feeding of GM corn has no significant effect on milk production and milk composition (Barriere et al., 2001; Calsamiglia et al., 2007; Clark and Ipharraguerre, 2001; Donkin et al., 2003; Grant et al., 2003; Ipharraguerre et al., 2003). Beyond that, possible effects of GM feed on further animal performance were thoroughly investigated (Flachowsky et al., 2005). Recombinant DNA was not detected in milk of cows fed genetically modified feed (Calsamiglia et al., 2007; Nemeth et al., 2004; Phipps et al., 2002; Phipps et al., 2003; Phipps et al., 2005). Despite the finding, that DNA fragments can cross the intestinal barrier, neither fragments of *cry1Ab* DNA nor the Cry1Ab protein were detectable in blood (Bertheau et al., 2009). However, in dairy cows, only short-term experiments with a limited number of animals have been conducted so far, and no results are available on potential long-term effects.

The objective of this study was to investigate the potential transfer and fate of *cry1Ab* DNA and immunoreactive fragments of the Cry1Ab protein from feed into blood, milk, feces and urine of

dairy cows fed genetically modified corn in a long-term study lasting 25 months using sensitive, highly specific and validated detection and quantification methods.

MATERIAL AND METHODS

Long-term Feeding Experiment

36 lactating Fleckvieh cows were housed at the Bavarian State Research Center for Agriculture (Germany) for 25 months and separated into two groups according to performance parameters and lactation period number. The target group (N=18, nine primiparous, nine multiparous) was fed on a ration containing genetically modified corn (MON810), and the control group (N=18, nine primiparous, nine multiparous) was fed on a ration based on the non-transgenic variety, with a mean daily DMI of 18.6 ± 3.6 kg and a mean daily milk yield of 24.5 ± 7.2 kg among all cows. The feed composition is shown in Table 1. All animals had access to feed and water ad libitum. According to the milk yield, further concentrates (41.2% corn kernels, 34.4% rapeseed meal, 19.9% molasses dried beet pulp, 3.2% mineral mixture and 2.2% rape oil) were offered above a milk yield of 22 kg d^{-1}. Cows with a milk yield below 18 kg per day were fed a partial total mixed ration (PTMR) mixed with 27.7 % straw, whereas the dry ration contained a PTMR mixed with 35.7 % straw. Nine cows of each group were exchanged and replaced with heifers due to illness or infertility. The GM corn and the non-transgenic variety had been cultivated and harvested in 2004, 2005 and 2006 under similar agronomic conditions at the Bavarian State Research Center for Agriculture (Germany). Nutrient and energy contents were comparable, ensuring equivalent feed conditions (data not shown).

The study was performed under the approval of the Bavarian State Research Center (LfL, Grub, Germany) institutional animal care and use committee.

Sampling

Representative feed samples (corn kernels, corn cobs, corn silage and PTMR) were collected weekly and ground in liquid nitrogen using mortar and pestles. Samples from each feed component of four experiment weeks were pooled for analysis. Monthly, milk samples were taken

manually from each cow before milking at 6 a.m. (15.1 ± 6.7 samples per cow over 25 months), whereas jugular blood (27 mL, Vacuette K3E/EDTA, Greiner Bio-One, Austria) and feces samples were taken monthly after milking. Spontaneous urine samples were taken bimonthly during the morning. Samples were stored at -20°C until used for DNA and protein extraction.

DNA Analysis

DNA Extraction of Feed and Feces

An optimized extraction procedure (Guertler et al., 2009) was applied to isolate genomic DNA from all feces and pooled feed samples (corn kernels, corn cobs, corn silage and PTMR). Feces or ground feed (300 mg fresh weight) was used for DNA extraction and filled in FastPrep Tubes containing 300 mg matrix beads (Lysing Matrix D, MP Biomedicals, Germany). After addition of CTAB extraction buffer the mixture was homogenized by applying the bead-beating FastPrep technique (FP120, MP Biomedicals, Germany) at 5.5 m s^{-1} for 30 s. After addition of 40 µL Proteinase K (20 mg mL^{-1}), the samples were incubated on a shaker over night at 60°C. Further, a centrifugation step at 16,000 × g and 4°C for 15 min was performed and the supernatant was transferred into a new tube. After RNA digestion by addition of RNase A (10 mg mL^{-1}) and subsequent centrifugation, the supernatant was filled into a new tube. Chloroform (200 µL) was added, followed by vigorous shaking and centrifugation for 10 min at 16,000 × g and 4°C. The upper watery phase was applied to 500 µL 2-propanol (80%) and 2 µL glycogen (20 mg mL^{-1}, Sigma, Germany). The samples were incubated for 1 h at room temperature and subsequently centrifuged at 16,000 × g for 10 min. The DNA pellet was dissolved in EtOH (70%) and loaded on a Wizard SV Column (Promega, Germany). The column was centrifuged and washed using 2-propanol (80%). Finally, 50 µL of prewarmed Tris-HCl (10 mM, pH=9.0, 70°C) was used for DNA elution.

DNA Extraction of Blood

Genomic DNA from blood was extracted using the Invisorb Blood Universal Kit (Invitek, Germany) following the manufacturer's protocol. All buffers and enzymes were included in the kit. One mL of whole blood was added to 2.5 mL Buffer EL to lysate the erythrocytes and placed on ice for 10 min after mixing. After centrifugation at 1,000 × g for 5 min, the supernatant was removed and again 2.5 mL Buffer EL were added. The sample was vortexed shortly and centrifuged at 1000 × g for 5 min. The supernatant was removed and the tube was placed upside down on a paper tissue to remove the residual fluid. Cells were lysed by adding 0.5 mL Buffer HL and 5 µL Proteinase K, followed by an incubation step of 10 min at 60°C while shaking. Precipitation solution (0.5 mL) was filled in a 15 mL tube and the lysate was added. After mixing, the tube was centrifuged at 2,000 × g for 5 min and the supernatant was carefully removed. The tube was inverted and placed on paper tissue to remove the residual fluid. Traces of ethanol were evaporated by air-drying the DNA pellet for 5 min. The DNA was dissolved by adding 0.2 mL Buffer U and incubation at 60°C for 1 hour.

DNA Extraction of Urine

PeqGOLD Trifast was used to isolate genomic DNA from urine samples. One mL Trifast was added to 100 µL urine and the mixture was incubated at room temperature (RT) for 5 min. Chloroform (200 µL) was added and the tube was vortexed vigorously, followed by incubation for 10 min at room temperature. After centrifugation at 12,000 × g and 4°C for 10 min, three layers were visible. The watery layer contains RNA whereas the inter-layer and the phenol-chloroform layer contain DNA and proteins. After removing the watery layer, 300 µL of ethanol (100%) were added to precipitate the DNA. The tube was inverted 2-3 times and stored at RT for 3 min, followed by centrifugation at 2,000 × g and 4°C for 5 min. Washing of the DNA pellet was performed by adding 1 mL of sodium citrate (0.1 M in 10% ethanol) and incubation for 30 min at RT. After centrifugation for 5 min at 2,000 × g and 4°C the supernatant was removed and the pellet washed again with sodium citrate. Subsequently, the DNA pellet was dissolved in 2 mL

153 ethanol (75%) and incubated for 15 min at room temperature. After mixing the solution, an
154 incubation step was performed at 2,000 × g and 4°C for 5 min. Finally, the pellet was dried and
155 dissolved in 50 µL NaOH (8 mM). The pH was adjusted to 8.4 by addition of 0.1 M HEPES.

156 ***DNA extraction of milk***

157 For genomic DNA extraction from whole milk, a guanidine hydrochloride based isolation
158 protocol (Guertler et al., 2009) was applied. Briefly, 300 µL of whole milk were added to 860 µL
159 extraction buffer and 100 µL guanidinehydrochloride solution. Proteinase K was used for protein
160 digestion. After incubation overnight at 60°C and subsequent centrifugation, the clear supernatant
161 was transferred to a new tube and RNase A was added, followed by an incubation step. The
162 sample was loaded on Wizard SV Columns (Promega, Germany) and centrifuged for 2 min at
163 10,000 × g and 4°C. The flow-through was discarded and the column was washed twice using 2-
164 propanol (80%). The DNA was eluted with 50 µL Tris-HCl (10 mM, ph=9.0, prewarmed at 70°C)
165 and stored at -20°C until used.

166 ***Qualitative end-point PCR***

167 DNA concentration of all samples was determined by using a Biophotometer (Eppendorf,
168 Germany). Additionally, the DNA purity was obtained by using the UV absorption ratios of
169 260/280nm and 260/230nm.

170 Qualitative end-point PCR was performed to amplify a small fragment of *cry1Ab* (206 bp,
171 Accession No. AY326434) in blood, milk, feces, urine and feed. Further, as a positive DNA
172 extraction control, a 173 bp fragment of *rubisco* (*ribulose-1,5-bisphosphate*
173 *carboxylase/oxygenase*, Accession No. X86563) and a 354 bp fragment of *GAPDH*
174 (*glyceraldehyde-3-phosphate dehydrogenase*, Accession No. NM_001034034) (milk, blood and
175 urine only) were amplified. Primer sequences and PCR design are published in detail elsewhere
176 (Guertler et al., 2007; Guertler et al., 2009). Isolated DNA from GM corn (MON810) served as a
177 positive control for *cry1Ab* and *rubisco*, while DNA from bovine liver served as a positive control

178 for *GAPDH*. Water was included as a negative control. The PCR product was separated by gel
179 electrophoresis and visualized by staining with GelRed (Biotium, USA).

180 ***Quantitative real-time PCR (qPCR)***

181 The LightCycler system with SYBR Green (Roche Diagnostics, Germany) was applied to
182 amplify a 206 bp fragment of *cry1Ab* in feed and milk samples. The qPCR MasterMix consisted
183 of 1 µL LC FastStart DNA Master SYBR Green I (Roche Diagnostics, Germany), 4 pM of each
184 primer, 3 mM $MgCl_2$ and water, resulting in a final volume of 10 µL. The total amount of
185 genomic DNA added to each qPCR reaction was 50 ng. A total of 40 cycles was completed under
186 the following cycling conditions: initial denaturation at 95°C for 10 min to activate the DNA
187 polymerase and to ensure complete denaturation of the DNA, denaturation at 95°C for 15 s,
188 annealing at 60°C for 10 s and extension at 72°C for 25 s. Unspecific signals were removed by
189 adding a forth step at 86°C for 5 s before fluorescence acquisition. A melting curve analysis was
190 performed to verify the correct and selective amplification of the amplicon. Furthermore, PCR
191 products were commercially sequenced to verify the specificity of the amplification. Samples were
192 measured in duplicates and analyzed using the standard curve method. A standard curve for feed
193 samples was generated by dilution of genomic DNA obtained from GM corn (MON810) to
194 concentrations ranging from 10 copies to 10^4 copies of *cry1Ab* μL^{-1} (Figure 1). For the milk
195 samples, a standard curve was created by spiking whole milk with predefined copy numbers of
196 *cry1Ab* (Guertler et al., 2009). Copy numbers were calculated following the formula (genomic
197 DNA concentration in pg $\mu L^{-1} \times 6.0233 \times 10^{23}$ copies mol^{-1})/(haploid corn genome (bp) × 660 ×
198 10^{12}) (Mitchell et al., 2009). A genome size of 2.5 Mbps for the haploid corn genome was used
199 (Arumuganathan and Earle, 1991).

200 Intra-assay and inter-assay coefficients of variation were determined by analyzing three samples
201 from the standard curves containing different copy numbers of *cry1Ab* (for feed 10,000, 500 and
202 50 copies μL^{-1}, for milk 10,000, 100 and 10 copies μL^{-1} after spiking).

Protein Analysis

Reagents

Reagents used were of analytical grade and supplied by Merck (Darmstadt, Germany) unless specified otherwise. HPLC-purified trypsin-activated Cry1Ab protein for standard preparation was generously provided by Dr. William J. Moar, Auburn University, USA.

Highly specific polyclonal antibodies were raised in rabbits through immunization, affinity purified and labeled with biotin as described elsewhere (Paul et al., 2008).

Cry1Ab Protein Analysis in Feed

The Cry1Ab protein was extracted by homogenization of ground pooled feed samples using the FastPrep FP120. 100 mg of each sample, mixed with 1 mL of ice-cold extraction buffer (8 mM sodium phosphate, 137 mM NaCl, 2.7 mM KCl, 1.5 mM potassium phosphate containing 0.1% Tween 20 and protease inhibitors, pH 7.4), were transferred to 2 mL FastPrep tubes filled with 200 mg matrix beads, and homogenized five times. Each homogenization cycle was performed at 6 m s^{-1} for 30 s, followed by an incubation step of 10 min on ice. Finally, a clear sample extract was collected after centrifugation of the homogenate at 15,000 × g and 4°C for 15 min. Transgenic feed extracts (corn kernels and cobs) were diluted 1:5 with extraction buffer before analysis.

A previously developed and optimized sandwich ELISA for Cry1Ab protein quantification was validated following the guidelines of the European Commission Decision 2002/657/EC and used for the analysis of all feed ingredients and PTMR sample extracts (Paul et al., 2008). In brief, the assay was performed in 96-well microtiter plates (Maxisorp™, Nunc, Denmark) coated with the immunoaffinity purified anti-Cry1Ab protein rabbit polyclonal antibody (capture antibody) at a concentration of 0.025 µg $well^{-1}$ in 100 µL coating buffer. The plates were incubated overnight at 4°C under gentle shaking. After 12 h, the contents of the coated plates were decanted and remaining unbound active sites on each well were blocked by incubating 300 µL of 1% bovine serum albumin (BSA; SERVA, Heidelberg, Germany) in PBST (8 mM sodium phosphate, 137

228 mM NaCl, 2.7 mM KCl, 1.5 mM potassium phosphate containing 0.1% Tween 20 pH 7.4) per
229 well for 1 h at RT while shaking. After decantation of the blocking buffer, the capture antibody
230 coated and blocked plates were stored frozen at –20°C until used. At the time of assay, frozen
231 plates were thawed and washed twice with assay buffer. Aliquots of 50 µL Cry1Ab protein
232 calibrators (0.1 ng mL^{-1} to 20 ng mL^{-1}), controls (0.2, 4 and 16 ng mL^{-1}) and unknown feed sample
233 extracts (transgenic corn cobs and corn kernels extracts diluted with extraction buffer 1:5) were
234 added to respective wells in duplicates followed by the addition of 100 µL assay buffer (PBST
235 containing 0.1% BSA, pH 7.4). Plates were incubated overnight at 4°C while shaking, and washed
236 six times with assay buffer on the next day. Then, 100 µL biotinylated detection antibody (5 ng
237 mL^{-1} diluted in assay buffer) was added to each well, and incubated at RT for 1 h on a plate
238 shaker. After four washing steps with assay buffer, streptavidin-horseradish peroxidase enzyme
239 conjugate (Roche Diagnostics, Germany; 100 µL, diluted 1:15000 times in assay buffer) was
240 added to each well and incubated for 15 min at RT. After four washing steps, 150 µL 3,3',5,5'-
241 tetramethylbenzidine (TMB; Sigma, Germany) enzyme substrate solution was added to each well
242 and the plates were incubated for 40 min at RT in the dark. Thereafter, the enzymatic reaction was
243 stopped by addition of 2 M sulfuric acid (50 µL $well^{-1}$) and the absorbance was read at 450 nm in
244 a microplate reader (Sunrise, Tecan, Germany). The calibration curve for Cry1Ab protein was
245 constructed using online Magellan V6.1 software (Tecan, Austria) and the concentration of
246 Cry1Ab protein in unknown samples was determined by interpolation. All data are presented as ng
247 of Cry1Ab protein g^{-1} DM of feed sample.

248 ***Cry1Ab Protein Analysis in Feces***

249 100 mg (wet weight) feces were extracted with 1 mL ice-cold extraction buffer (8 mM sodium
250 phosphate, 137 mM NaCl, 2.7 mM KCl, 1.5 mM potassium phosphate, 2.5mM EDTA containing
251 0.5% skim milk powder, 0.1% Tween 20 and protease inhibitors, pH 7.4) and 200 mg matrix
252 beads after homogenization (six times) and subsequent centrifugation (15,000 × g at 4°C for 15

253 min). Extracts were stored at -20°C until analyzed. 50 µL sample extract was used to quantify
254 Cry1Ab protein applying the ELISA and results are presented as ng of Cry1Ab protein g^{-1} wet
255 weight of feces.

256 ***Cry1Ab Protein Analysis in Blood***

257 An ELISA for the detection of Cry1Ab protein was previously optimized (Paul et al., 2008).
258 The data are presented as ng Cry1Ab protein mL^{-1} blood plasma.

259 ***Cry1Ab Protein Analysis in Urine***

260 Frozen urine samples were thawed and centrifuged at 4,000 × g for 10 min at 4°C. 40 µL of
261 clear urine supernatant was used in the ELISA to quantify the Cry1Ab protein. A matrix-matched
262 calibration curve was generated by preparing Cry1Ab protein calibrators (0.1 to 25 ng/mL of urine
263 free from Cry1Ab protein). The results are presented as ng of Cry1Ab protein mL^{-1} of urine.

264 ***Cry1Ab protein analysis in milk***

265 Skim milk was separated from the whole milk by centrifugation at 3,000 × g and 4°C for 15
266 min and 40 µL were further used in the ELISA. The ELISA procedure is described in detail
267 elsewhere (Guertler et al., 2009). The data are presented in ng Cry1Ab protein mL^{-1} skim milk.

268 ***Optimization and validation***

269 ELISA assays were validated as presented in previous publications (Guertler et al., 2009; Paul
270 et al., 2008).

271 ***Statistics***

272 The decision limit (CCα) and the decision capability (CCβ) of the ELISA were determined
273 according to the guidelines of the European Commission Decision 202/657/EC (European
274 Commission, 2002) as described in detail elsewhere (Paul et al., 2008). In brief, three times the
275 mean signal of noise level recorded for Cry1Ab protein in blanks is equal to CCα. CCβ is
276 determined using the equation $CC\beta = CC\alpha + 1.64 \times S.D.s$. S.D.s is the standard deviation obtained
277 for blank samples fortified at concentrations of CCα.

All samples are presented as mean of all cows (target or control group) per month ± SEM.

RESULTS

Feed

DNA extraction from feed resulted in concentrations ranging from 42 to 970 ng DNA μL^{-1} of isolate for corn kernels, from 49 to 359 ng DNA μL^{-1} isolate for corn cobs, from 41 to 163 ng DNA μL^{-1} isolate for corn silage and from 38 to 197 ng DNA μL^{-1} isolate for PTMR. The photometrical analysis revealed a DNA purity suitable for PCR analysis, as 260/280 ratios were between 1.60 and 1.98.

By means of qPCR, 37 copies of *cry1Ab* DNA g^{-1} DM were reproducibly detectable. The suitability of the extraction and quantification procedure is underlined by a mean intra-assay CV of 0.15 (n=9) and a mean inter-assay CV of 0.20 (n=3, six replicates each (Table 2)). A melting curve analysis and subsequent sequence analysis ensured the specificity of the amplicon (data not shown).

Protein analysis revealed a CCα of 1.35 ng g^{-1} and a CCβ of 2.08 ng g^{-1} corn kernels, a CCα of 1.32 ng g^{-1} and a CCβ of 1.65 ng g^{-1} corn cobs, a CCα of 4.6 ng g^{-1} and a CCβ of 5.61 ng g^{-1} corn silage, a CCα of 8.18 ng g^{-1} and a CCα of 12.26 ng g^{-1} PTMR. The assay precision is illustrated by a mean intra-assay CV of 3.57 (n=8, three replicates each) and a mean inter-assay CV of 7.94 (n=8, three replicates each; Table 2). In corn kernels, a mean recovery of 88.6% was achieved, 74.7%, 76.5% and 78.9% mean recovery rates were achieved for corn cobs, corn silage and PTMR, respectively (Table 3).

In all feed samples of the long-term experiment (corn kernels, cobs, silage and PTMR), specific amplicons of *rubisco* (173bp) were amplified by conventional PCR. In non-transgenic feed samples, no *cry1Ab* fragment (206bp) or immunoreactive fragment of the Cry1Ab protein (all samples were below CCα) was detectable by using qPCR and ELISA. Only in non-transgenic corn kernels, two samples were found above the CCα, which is most likely due to sampling or post-

303 sampling contamination. Transgenic corn kernels, corn cobs and PTMR were positive for
304 recombinant *cry1Ab* DNA. However, due to rapid degradation of DNA during the ensiling
305 process, only two samples of transgenic corn silage were positive for *cry1Ab*. In all transgenic
306 feed samples, immunoreactive fragments of the Cry1Ab protein were present. The range of
307 *cry1Ab* DNA and Cry1Ab protein, detectable by qPCR and ELISA in feed samples of the long-
308 term experiment, is depicted in Table 4.

309 During the first lactation, the mean Cry1Ab protein intake was 6.0 mg kg^{-1} DM among cows fed
310 GM corn, whereas the mean Cry1Ab protein intake during the second lactation was 6.1 mg kg^{-1}
311 DM (Steinke et al., 2009). To the best of our knowledge, this is the first intake balancing on
312 Cry1Ab protein. Previous publications did not provide information about the actual amount of
313 Cry1Ab protein intake.

314 ***Feces***

315 Isolation of DNA from feces resulted in concentrations above 50 ng DNA μL^{-1} of isolate and
316 integrity values between 1.6 and 1.8. A small fragment of *rubisco* was amplified as a positive
317 extraction control and was detected in all feces samples of the long-term experiment. The *cry1Ab*
318 DNA could not be amplified by means of conventional PCR at a limit of detection (LOD) of
319 17,000 copies g^{-1} feces (wet weight) (Figure 2).

320 Protein analysis revealed no sample of the control group above the CCα of 1.21 ng g^{-1}. The
321 concentration of Cry1Ab protein in samples of the target groups ranged from 20 to 110 ng g^{-1}
322 (Figure 3 A and B).

323 ***Blood***

324 Genomic DNA from bovine blood was successfully isolated and DNA concentrations from 40 to
325 800 ng DNA μL^{-1} of isolate were measured. The high purity of DNA was underlined by
326 260/280nm ratios between 1.5 and 1.9. Extracted DNA was analyzed using conventional PCR

amplifying a 354bp fragment of *GAPDH* as a positive extraction control, a 173bp fragment of *rubisco* and a 206bp fragment of *cry1Ab*.

Immunoreactive fragments of Cry1Ab were analyzed by applying an optimized ELISA with a CCα of 1.53 ng mL^{-1} and a CCβ of 2.3 ng mL^{-1} (Paul et al., 2008). Recoveries ranged from 89 to 106%.

In all blood samples, *GAPDH* DNA was detected, whereas fragments of *rubisco* and *cry1Ab* were absent within the LOD of 10^5 copies of *cry1Ab* DNA mL^{-1} blood (Figure 4). Cry1Ab protein analysis revealed that all blood plasma samples were below the CCα of 1.53 ng mL^{-1} (Figure 3 C and D). There was no difference between the target and the control group.

Urine

Concentrations of genomic DNA isolated from bovine urine varied from 6 to 40 ng DNA μL^{-1} of isolate with 260/280 ratios between 1.3 and 1.7. Conventional PCR was applied to amplify a 206 bp fragment of *cry1Ab*. No urine sample of the long-term experiment was found positive for novel DNA at a LOD of 23,000 copies mL^{-1}. *GAPDH* was used as an extraction control and was amplified in most of the urine samples.

For the Cry1Ab protein, all samples were below the CCα of 0.3 ng mL^{-1}, except one sample of month 5 showing a protein concentration of 1.59 ng mL^{-1}, which is most likely due to a contamination, also expressed by a high SEM of 0.297 (Figure 3 E and F).

Milk

Genomic DNA isolation revealed concentrations between 45 and 250 ng DNA μL^{-1} of isolate, with a 260/280nm ratio of 0.9 to 1.8.

The analytical range for *cry1Ab* DNA in whole milk was 10^5 to 10^8 copies mL^{-1} with a mean recovery rate of 84.9 % (n=3, six replicates each), an intra-assay CV of 0.15 (n=9) and an inter-assay CV of 0.78 (n=9, three replicates each) (Guertler et al., 2009).

Validation of the Cry1Ab protein ELISA for skim milk resulted in a CCα of 0.25 ng mL^{-1} and a CCβ of 0.4 ng mL^{-1}. The recovery values ranged from 88 to 104% (Guertler et al., 2009).

By using conventional PCR, a 354bp fragment of *GAPDH* (positive extraction control) was amplified in all milk samples of the long-term experiment, whereas fragments of *rubisco* (173bp) and *cry1Ab* (206bp) were absent within the LOD. No *cry1Ab* DNA was detected above the LOD of 100 copies μL^{-1} by means of qPCR. Analysis of skim milk using an ELISA resulted in values below the CCα of 0.25 ng ml^{-1} (Figure 3 G and H), showing no differences between the control group and the target group.

DISCUSSION

This study was accomplished to investigate the fate of novel DNA and Cry1Ab protein during the metabolism of the dairy cow after long term feeding of genetically modified corn (MON810). Sensitive and specific assays were developed and validated according to the European Commission Decision 2002/657/EC and the "Minimum Requirements for Analytical Methods of GMO Testing" published by the European Network of GMO Laboratories (ENGL).

Regarding the feed analysis, results of the qPCR and the ELISA revealed that non-transgenic feed was free of recombinant DNA and the novel protein within the LOD. In corn kernels, two samples were found positive for the Cry1Ab protein (above CCβ), which might be caused by sampling or post-sampling contamination. In all samples of the transgenic feed, fragments of *cry1Ab* DNA and the Cry1Ab protein were detected, except for corn silage, in which novel DNA at a fragment length of 206bp was only detected in two sets of samples. The minimal ability to detect DNA in silage samples may be caused by the ensiling process, which leads to rapid degradation of DNA (Hupfer et al., 1999; Lutz et al., 2006), and due to the fermentation in the bovine rumen, where no fragments of GM DNA were detected in the liquid phase of the rumen or in duodenal contents (Phipps et al., 2003). These results indicate, that most of the ingested Cry1Ab protein and *cry1Ab*

375 DNA originate from corn cobs. Cry1Ab expression is variable among different corn tissues
376 (Hubert et al., 2008).

377 The fate of *cry1Ab* DNA and Cry1Ab protein after feeding GM corn to dairy cows was
378 investigated over a period of 25 months. Fragments of the highly abundant *GAPDH* (in blood,
379 urine and milk samples) or *rubisco* genes (in feed and feces samples), which served as positive
380 extraction controls, were found in each sample, underlining the suitability of the extraction and
381 detection methods applied for DNA analysis. The suitability of GAPDH as a positive control for
382 extraction from milk and blood is often described (Leutenegger et al., 2000; Robinson et al.,
383 2007). In feces, blood and urine, a 206bp fragment of *cry1Ab* could not be detected by means of
384 PCR, which is in accordance to other studies (Einspanier et al., 2001; Nemeth et al., 2004; Phipps
385 et al., 2003). On the one side, it is reported, that DNA fragments are able to cross the intestinal
386 barrier into the blood stream (Alexander et al., 2007). On the other side, a rapid degradation
387 throughout the gastrointestinal tract takes place (Wiedemann et al., 2006), which might explain the
388 absence of novel DNA in blood after feed intake. The low number of copies of *cry1Ab* which
389 might cross the intestinal barrier into the blood stream would also impede the tracing of a possible
390 transfer of recombinant DNA into the blood stream (Bertheau et al., 2009). As there is no GM
391 DNA persistence in the blood in detectable levels, a transfer of GM DNA into urine is highly
392 unlikely, which supports the absence of *cry1Ab* DNA in urine samples within the LOD. It is also
393 stated that urea is an inhibitory component (Khan et al., 1991), preventing an amplification by
394 means of PCR. Milk samples were shown to be free of *cry1Ab* by applying conventional PCR.
395 These results were supported by quantification of the *cry1Ab* DNA using qPCR, showing no
396 sample above the LOD. In several studies, the absence of recombinant DNA in milk was stated
397 (Phipps et al., 2002; Phipps et al., 2005; Poms et al., 2003). However, small fragments of
398 recombinant DNA were found in milk samples from the Italian market, which the authors traced
399 back to a fecal contamination (Agodi et al., 2006).

Regarding the Cry1Ab protein, feces samples of the control group were free of novel protein, whereas the Cry1Ab protein was found in all feces samples of the target group. This finding goes in line with the known limited digestibility of corn crude protein (Romagnolo et al., 1994). Concerning the quantification of Cry1Ab in blood, no sample showed signals above the CCα. The absence of Cry1Ab in blood is supported by the findings, that the novel protein is degraded in the bovine rumen (Einspanier et al., 2004; Lutz et al., 2005; Wiedemann et al., 2006). The lack of absorption mechanisms for the transfer of Cry1Ab protein into the blood stream might also be a reason for the absence of Cry1Ab protein in the blood. Urine samples were free of Cry1Ab protein, except of one sample. This is most likely caused by fecal contamination during the sampling process. In accordance to previous findings (Calsamiglia et al., 2007), Cry1Ab quantification in milk revealed no sample above the CCα.

In conclusion, after feeding genetically modified corn (MON810) to dairy cows for 25 months, no transfer of recombinant DNA or Cry1Ab protein into the animals' circulation was detected. To the best of our knowledge, this large scale study with 36 lactating cows is the first long-term investigation on the fate of recombinant DNA and the Cry1Ab protein. For DNA extraction and quantification of the DNA and immunoreactive fragments of the Cry1Ab protein, new sensitive and highly specific assays were established and thoroughly validated according to existing guidelines of GM organism testing. In contrary to previous studies, whole milk was used for the quantification of *cry1Ab* DNA and the Cry1Ab protein in milk. The results of this study revealed a rapid degradation of *cry1Ab* DNA and novel protein after feed intake. However, undigested corn particles may pass the GIT resulting in a detectibility of the Cry1Ab protein in feces of cows fed GM corn.

IMPLICATIONS

A mean daily intake of Cry1Ab protein of 6.1 mg kg^{-1} DM was observed for cows fed GM corn, which is the first balancing of Cry1Ab protein intake. Anyhow, *cry1Ab* DNA and Cry1Ab protein

425 are not detectable in whole milk – even not with most sensitive methods. Hence, milk of dairy
426 cows fed genetically modified corn for 25 months is classified not different from milk of cows fed
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TABLES

Table 1: Feed composition of the daily animal diet

Component	Ratio, DM
Corn silage[a]	41.9%
Corn cobs[a]	21.2%
Grass silage	11.0%
Straw	5.9%
Molasses	1.4%
Concentrates	18.6%
• Rapeseed meal	• 51.1%
• Corn kernels[a]	• 41.2%
• Mineral mixture	• 5.3%
• Rape oil	• 2.4%

[a] Ingredients contained 100% MON810 in GM ration and conventional corn in non-GM ration

Table 2: Analytical precision for quantitative real-time PCR and ELISA in transgenic feed (corn kernels). Three different concentrations C1, C2 and C3 were used to determine the coefficients of variation (CV).

Coefficient of variation (CV)[a]	Quantitative real-time PCR (DNA)				ELISA (Protein)			
	extracted *cry1Ab* controls			Mean CV	spiked Cry1Ab controls			Mean CV
	C1 10^4 copies/µL	C2 500 copies/µL	C3 50 copies/µL		C1 800 pg/well	C2 200 pg/well	C3 10 pg/well	
Intra-assay	0.03	0.06	0.36	**0.15**	2.54	1.76	6.42	**3.57**
Inter-assay	0.17	0.19	0.24	**0.20**	4.12	5.32	14.39	**7.94**

[a] Coefficients of variation at different concentrations of *cry1Ab* DNA (three determinants per assay; in total 7 independent assays) and Cry1Ab protein (8 independent assays; each assay with three replicates of each control)

560

561 Table 3: Recoveries achieved for Cry1Ab protein in spiked feed samples

	Cry1Ab protein		
	Amount added, ng g^{-1}	Amount measured, ng g^{-1}	Recovery
Corn kernels	1.60	1.72 ± 0.17	107.8
	16.73	14.18 ± 0.95	84.8
	69.05	55.48 ± 1.73	80.3
	138.25	112.50 ± 8.25	81.4
	Mean Recovery		**88.6**
Corn cobs	1.60	1.35 ± 0.12	84.6
	17.75	13.61 ± 1.15	76.7
	72.04	50.16 ± 4.57	69.6
	134.37	90.97 ± 12.91	67.7
	Mean Recovery		**74.7**
Corn silage	4.72	3.61 ± 0.35	76.5
	16.53	12.24 ± 0.89	74.1
	69.35	47.88 ± 6.67	69.0
	109.28	94.38 ± 8.40	86.4
	Mean Recovery		**76.5**
PTMR	5.14	5.08 ± 0.62	98.8
	18.81	13.87 ± 0.576	73.7
	63.98	46.47 ± 3.05	72.6
	137.21	96.89 ± 7.16	70.6
	Mean Recovery		**78.9**

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565 Table 4: Amount of *cry1Ab* DNA and Cry1Ab protein detected in different feed components (n.d. = not detected)

Feed component	**Cry1Ab protein non-transgenic, ng g^{-1} DM**	**Cry1Ab protein transgenic, ng g^{-1} DM**	**Cry1Ab protein Assay CCα, ng g^{-1} DM**	**Cry1Ab protein Assay CCβ, ng g^{-1} DM**	***cry1Ab* DNA non-transgenic, copies g^{-1} DM**	***cry1Ab* DNA transgenic, copies g^{-1} DM**	***cry1Ab* DNA LOD[2], copies g^{-1} DM**
Corn kernels	< CCα[1]	155-379	1.35	2.03	n.d.	$5.6\times10^{4} - 7.1\times10^{6}$	37
Corn cobs	< CCα	226-1020	1.32	1.65	n.d.	$1.0\times10^{5} - 5.3\times10^{6}$	37
Corn silage	< CCα	91-390	4.60	5.61	n.d.	$0 - 8.3\times10^{4}$	37
PTMR	< CCα	210-452	8.18	12.26	n.d.	$2.7\times10^{4} - 3.6\times10^{6}$	37

566 [1] Two samples were found above CCβ, which might be due to sampling or postsampling contamination

567 [2] LOD = limit of detection

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569

570 **FIGURE LEGENDS**

571 Figure 1: Standard curve of DNA extracted from corn kernels containing different copy numbers as used in
572 the qPCR. The standard curve shows linearity between 10 and 10,000 copies μL^{-1} of isolate.

573

574 Figure 2: (A) Amplification of a 173 bp fragment of *rubisco* as a positive extraction protocol in feces
575 samples from cows fed transgenic corn; (B) amplification of a 206 bp fragment of *cry1Ab* in feces samples
576 from cows fed transgenic corn. (positive control is marked with a "+", negative control is marked with a "-
577 "; 100 ng of DNA were used in the PCR, 10 µL of PCR product were loaded on the gel)

578

579 Figure 3: Analysis of Cry1Ab protein by ELISA. Data are presented as ng Cry1Ab protein g^{-1} or mL^{-1} ±
580 SEM. CCα is marked with a solid line, CCβ is marked with a broken line. Cry1Ab protein is detectable in
581 feces of transgenic fed cows (A), but not in feces samples of cows fed non-transgenic corn (B). In blood (C
582 and D), urine (E and F) and milk (G and H), all samples were below the CCα. Only in one urine sample
583 from transgenic fed cows, the Cry1Ab protein was detected, which is most likely due to fecal
584 contamination.

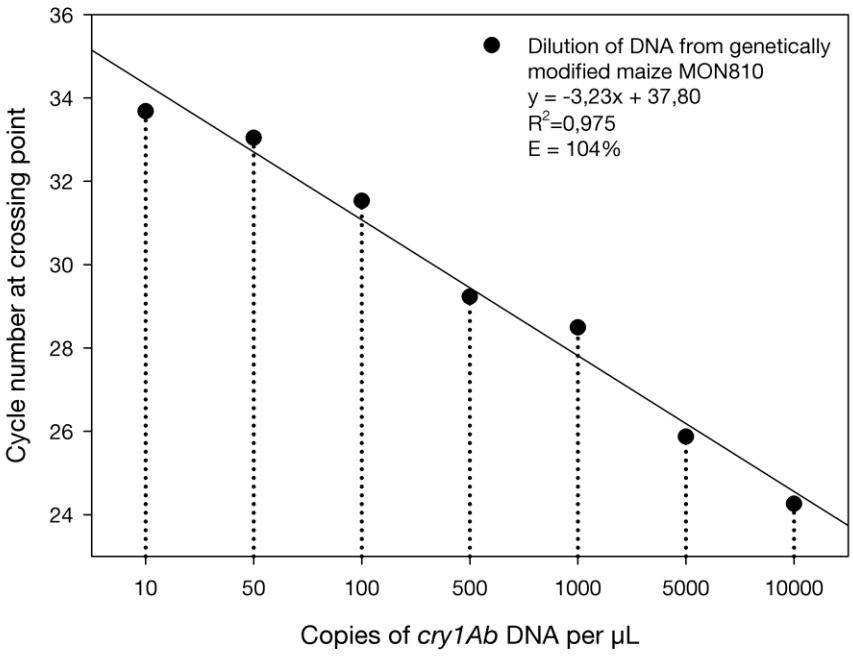
585

586 Figure 4: (A) Amplification of a 354bp fragment of *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH)*
587 as a positive extraction control in blood samples of cows fed genetically modified corn; (B) amplification
588 of a 173bp fragment of *rubisco*. The highly abundant chloroplast gene was not detected in blood samples;
589 (C) a 206bp fragment of *cry1Ab* was not detected in blood samples of the long-term experiment. (positive
590 control is marked with a "+", negative control is marked with a "-"; 100 ng of DNA were used in the PCR,
591 10 µL of PCR product were loaded on the gel)

592

593 **FIGURES**

594 Figure 1:



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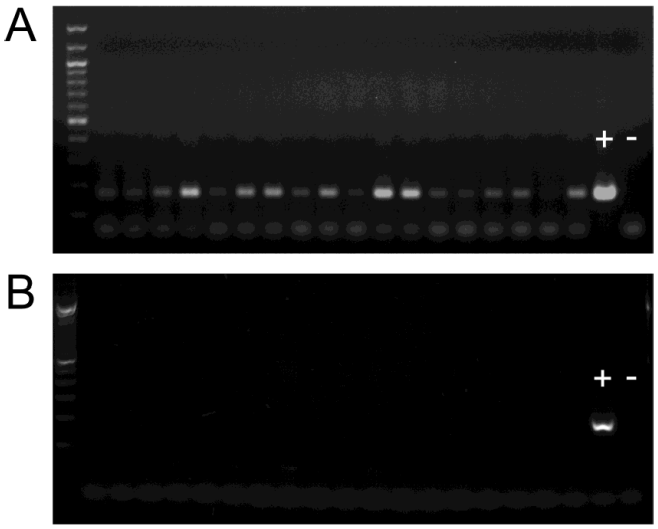
597 Figure 2



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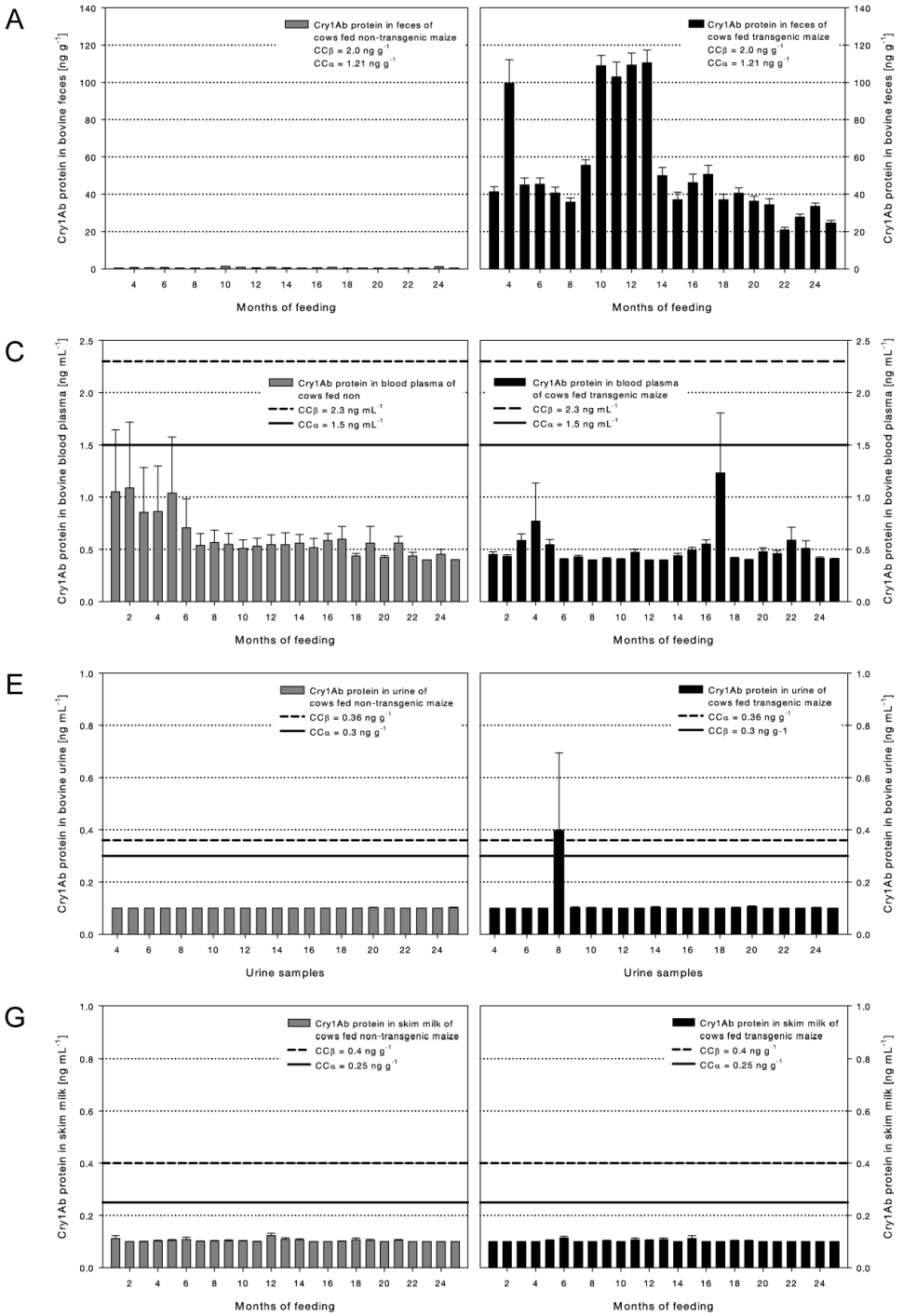
599 Figure 3:



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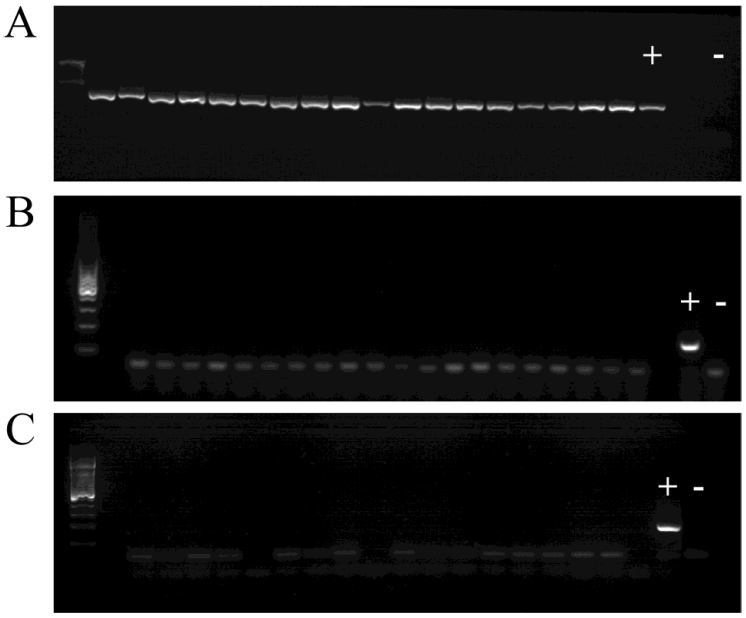
601 Figure 4:



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Appendix 3

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Short communication:

Degradation of Cry1Ab protein from genetically modified maize (MON810) in relation to total dietary feed proteins in dairy cow digestion

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Abstract

To investigate the relative degradation and fragmentation pattern of recombinant Cry1Ab protein from genetically modified (GM) maize MON810 throughout the gastrointestinal tract (GIT) of dairy cows, a 25 months GM maize feeding study was conducted on 36 lactating Bavarian Fleckvieh cows allocated into two groups (18 cows per group) fed diets containing either GM maize MON810 or nearly isogenic non-GM maize as the respective diet components. All cows were fed a partial total mixed ration (pTMR). During feeding trial, 8 feed (4 transgenic (T) and 4 non-transgenic (NT) pTMR) and respective 42 feces (26 T and 18 NT) samples from the subset of cows fed T and NT diets, and at the end of this feeding trial, digesta contents of rumen, abomasum, small intestine, large intestine and cecum were collected after the slaughter of six cows of each feeding group and analyzed for Cry1Ab protein and total protein using Cry1Ab specific ELISA and bicinchoninic acid (BCA) assay, respectively. Immunoblot analysis was performed to evaluate the integrity of Cry1Ab protein in feed, feces and digesta samples. A decrease of 44% in Cry1Ab protein concentrations from T pTMR versus the voided feces (9.40 versus 4.18 μg/g of total proteins) was recorded. Concentrations of Cry1Ab protein in GIT digesta of cows fed T diets varied between the lowest 0.38 μg/g of total proteins in abomasum to the highest 3.84 μg/g of total proteins in rumen. Immunoblot analysis revealed the extensive degradation of recombinant Cry1Ab protein into a smaller fragment of around 34 kDa in GIT. The results of the present study indicate that the recombinant Cry1Ab protein from MON810 is increasingly degraded into a small fragment during dairy cow digestion.

Keywords: Cry1Ab protein, dairy cow, genetically modified maize MON810, digesta

Introduction

Genetically modified (GM) maize MON810 expressing insecticidal Cry1Ab protein, has been approved as an animal feed in several countries worldwide. Already many studies on GM feeds have been documented regarding animal performance and health, as well as resultant food safety [reviewed by (Flachowsky et al., 2005; Report of the EFSA GMO panel working group on Animal feeding trials, 2008)]. However, open questions on the *in vivo* digestive degradation of transgenic protein in relation to other dietary feed proteins in ruminants still remain.

In general, dietary proteins are broken down to decreasingly smaller fragments under the influence of a sequence of processes that occur in different segments of the gastrointestinal tract (GIT) of ruminants. Those processes include fermentation of dietary components by microbes during incubation in the reticulo-rumen, acid hydrolysis and degradation by enzymes of the host animal in the abomasum and small intestine, respectively and secondary fermentation in the cecum and large intestine. Most of the ingested feed proteins are degraded (about 40 to 80%) in the rumen and utilized by rumen microbes for the synthesis of microbial protein [as reviewed by (Chalupa, 1975)]. However, maize proteins are relatively resistant to the rumen degradation (Romagnolo et al., 1994). Also the site of digestion affects the nature of absorbed end-products and the extent of nutrients losses occurring during digestion. Thus, the objective of the present study was to determine the Cry1Ab protein stability or degradation relative to total dietary proteins in feed, feces and GIT digesta of dairy cows.

Materials and methods

All animal handling and experimentation were conducted under the approval of the Bavarian State Research Center (LfL, Grub, Germany) institutional animal care and use committee. To investigate the digestive fate of recombinant Cry1Ab protein from genetically modified (GM) maize MON810 in dairy cows digestion, thirty-six Bavarian Fleckvieh cows were separated into a transgenic group (n=18) fed on a diet containing GM maize MON810 and a control or non-transgenic group (n=18) fed conventional maize over a period of 25 months. All experimental cows were housed at the Bavarian State Research Center and fed a partial total mixed ration (pTMR). The diet composition is shown in Table 1. According to the milk yield further concentrates (40.4% maize kernels, 34.4% rapeseed meal, 19.9% molasses dried beet pulp, 3.2% mineral mixture and 2.4% urea) were offered above 22 kg milk yield per day. To evaluate the digestive fate of recombinant Cry1Ab protein during the feeding trial, in total four samples of each diet (transgenic and non-transgenic maize kernel, cobs, silage, and pTMR) and respective forty-two feces (26 transgenic and 18 non-transgenic) samples were collected from the subset of cows. To determine the amount and fragmentation pattern of Cry1Ab protein in the GIT digesta, digesta contents of rumen, abomasum, small intestine, large intestine and cecum were collected after the slaughter of six cows of each

feeding group at the end of the feeding trial. All samples were stored at –80°C until analyzed for total protein and Cry1Ab protein.

All feed samples were finely ground in liquid nitrogen using mortal and pestles. Total proteins were extracted from the pulverized feed (100 mg), feces (100 mg) and GIT digesta (200 mg) samples after the homogenization with 1ml ice-cold extraction buffer (PBST; 8 mM sodium phosphate, 137 mM NaCl, 2.7 mM KCl, 1.5 mM potassium phosphate, 0.1% Tween 20 pH 7.4; containing protease inhibitors) using 300 mg matrix green ceramic beads in Fastprep homogenization machine. The supernatants were collected in 1.5 ml microcentrifuge tubes after centrifugation at 15,000 x *g* at 4°C for 15 min. A clear extract was collected after re-centrifugation at 15,000 x *g* at 4°C for 10 min and stored at –20°C until used for total protein and Cry1Ab protein quantification.

Total protein concentration in each extract was measured by bicinchoninic acid (BCA) assay (Smith et al., 1985) using BSA as protein standards. For determination of the Cry1Ab protein concentration a previously developed (Paul et al., 2008) sandwich ELISA was used after slight modifications and matrice specific assay validations (Guertler et al., 2009). Briefly, an assay was performed on Cry1Ab protein affinity purified specific antibody coated 96-well microtiter plates by incubating 50 µl of sample extract or Cry1Ab protein standards (HPLC-purified trypsin-activated Cry1Ab protein calibrator concentrations ranging from 2 to 1000 pg per 50 µl extraction buffer) along with 100 µl assay buffer (PBST containing 0.1% BSA). After overnight incubation at 6 to 8°C plates were washed six-times with 300 µl PBST per well. The amount of antibody captured Cry1Ab protein from samples/standards was measured by biotin labeled Cry1Ab specific antibody, streptavidin-peroxidase enzyme conjugate and 3,3',5,5'-tetramethylbenzidine (TMB) substrate reaction. Cry1Ab protein concentrations in unknown samples were interpreted from the Cry1Ab protein calibration curve generated using online software Magellan 6 (Tecan, Austria). The concentrations were finally presented as Cry1Ab protein µg per g total protein.

To monitor the fragmentation of Cry1Ab protein from MON810 in transgenic feed, feces and GIT digesta immunoblot analysis was performed. Total protein (60 µg total protein or 100 pg Cry1Ab protein extracts from T feed and feces or respective equivalent amount of total protein from NT feed and feces) of transgenic and non-transgenic feed, feces and GIT digesta were resolved on 12% SDS-PAGE, and transferred to nitrocellulose membranes (Protran AB 85, Whatman, Dassel, Germany). Membranes with

blotted protein bands were incubated with affinity purified anti-Cry1Ab protein (rabbit pAb, 0.1 μg/ml) (Paul et al, 2008), and HRP-labeled polyclonal goat anti-rabbit secondary (1:10000) antibody (Santa Cruz biotechnology, Germany). Antibody binding was visualized by chemiluminescence (Supersignal west pico system, Pierces, USA). In immunoblot analysis, a positive control containing HPLC purified trypsin activated Cry1Ab protein (65kDa) was used to confirm and verify the positive presence of Cry1Ab protein. Student's t-test was used to compare the means of Cry1Ab protein concentrations in transgenic feed and feces, and total proteins in GIT digesta of cows fed transgenic and non-transgenic diets. A *p*-value below 0.05 was considered significant.

Results and discussion

The results for Cry1Ab protein quantification using a validated sandwich ELISA (according to the guidelines cited in European Commission Decision 2002/657/EC) in feed (transgenic and non-transgenic pTMR) and feces samples of cows fed transgenic and non-transgenic diets showed a decrease in Cry1Ab protein concentration resulting in 44% ($p < 0.01$) in feces from cows fed transgenic pTMR. The mean levels of Cry1Ab protein in transgenic feed (pTMR) and feces were 9.40 and 4.18 μg Cry1Ab protein/g total protein, respectively (Fig 1). In contrast, the corresponding total protein contents in the feces (13.13 mg/g wet weight) of cows fed transgenic pTMR were 50% of the initial levels of the total dietary feed proteins (26.29 mg/g fresh weight). Digesta contents of rumen, abomasum, small intestine, large intestine and cecum of cows fed transgenic diets contained 3.84, 0.38, 0.83, 2.89 and 3.18 μg of Cry1Ab protein/g total protein, respectively (Fig 2). Corresponding samples of non-transgenic feed, feces and digesta were found below the assay decision limit (CCα) value of 1.21 ng Cry1Ab protein/g wet weight of the sample. The probable reason for higher concentration of Cry1Ab protein in rumen content could be the existence of relatively large particles of undigested feed and degradation of dietary proteins by rumen microbes. The lowest relative concentration of Cry1Ab protein in abomasum could be due to the release of microbial proteins into the abomasum. Further gradual increase in the relative Cry1Ab protein concentration in digesta of subsequent segments of the GIT (from abomasum to cecum) could be the due to absorption of end products of protein digestion in small intestine (amino acids and small peptides). The previous reports of feeding ruminants with other GM maize events; Bt11(Chowdhury et al., 2003) and Bt176 (Einspanier et

al., 2004; Lutz et al., 2005) have shown a similar degradation pattern of recombinant Cry1Ab protein in the bovine GIT. Although the concentrations of Cry1Ab protein measured in transgenic feed, feces and digesta of present investigation differs to those reported in these earlier studies. The reason for these differences in concentrations could be the different expression levels of recombinant protein in GM maize events (Bt11, Bt176 and MON810). The total protein concentrations showed a rising trend during the passage of the ingesta from rumen to cecum in the bovine GIT (Fig 3). However, no effect of two different diets (transgenic and non-transgenic) was observed on the total protein concentrations throughout the GIT. The results of immunoblot analysis for recording the fragmentation pattern of Cry1Ab protein from MON810 in transgenic feed, GIT digesta and feces revealed the degradation of full-sized (65kDa) recombinant protein into smaller immunoactive fragments of approximately 42, 34 and 17kDa (Fig 4-6). Fragments of 42kDa were present only in the transgenic feed (kernel, cobs and pTMR). The most prominent immunoactive fragment observed in all transgenic feed, digesta and feces was 34kDa. Week signals of 17kDa fragment were recorded in transgenic feed (kernel and cobs) and digesta (cecum). The probable reasons for the degradation of recombinant Cry1Ab protein are feed processing and storage, and microbial and enzymatic proteolysis in ruminant digestive tract. The previous reports (Lutz et al., 2005; Lutz et al., 2006; Wiedemann et al., 2006) of recombinant protein degradation from other GM maize (Bt176) in bovine GIT correspond with our present findings. The presence of only 34kDa fragment in feces could be attributed to the time-dependent degradation of Cry1Ab protein in rumen as suggested by the *in situ* degradation study on Cry1Ab protein in bovine rumen (Wiedemann et al., 2006). In the present study we could not detect any band of Cry1Ab protein and its fragments in transgenic silage, abomasal and small intestine digesta due to the low concentration (below the detection limit of 100 pg Cry1Ab protein for immunoblots) of recombinant protein in these samples.

In conclusion, the results of the present study indicate that Cry1Ab protein from MON810 is increasingly degraded during dairy cow digestion via small fragments of 42kDa, 34kDa and 17kDa. In comparison with total protein in feed the relative amount of Cry1Ab protein in feces is markedly reduced indicating that degradation of Cry1Ab protein seems to be faster than the degradation of total protein.

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1
2 **TABLES**
3
4 **Table 1. Ingredients and composition of the partial total mixed ration**
5

Ingredient	Ratio (DM basis)
Maize silage[1]	41.9%
Maize cobs[1]	21.2%
Grass silage	11.0%
Straw	5.9%
Molasses	1.4%
Concentrates	18.6%
• Rapeseed meal	• 51.1%
• Maize kernels[1]	• 41.2%
• Mineral mixture	• 5.3%
• Urea	• 2.4%

6 [1]Ingredients contained 100% MON810 in transgenic mixed ration and conventional maize in non-
7 transgenic mixed ration diets
8
9

FIGURE CAPTIONS

Fig 1. Concentration of Cry1Ab protein in feed (pTMR) and feces of dairy cows fed partial total mixed rations containing either transgenic maize or non-transgenic maize. * indicates ($p < 0.01$).

Fig 2. Relative Cry1Ab protein concentrations in different GIT digesta for cows fed a partial total mixed ration diets containing either transgenic or non-transgenic maize. Concentrations of Cry1Ab protein in non-transgenic diet fed digesta were below the assay decision limit (CCα) value of 1.21ng/g wet weight.

Fig 3. Relative total protein concentrations in different GIT digesta for cows fed a partial total mixed ration containing either transgenic or non-transgenic maize.

Fig 4. Western blot showing fragments of Cry1Ab protein in total protein extracts (60 μg) of transgenic and non-transgenic diet ingredients (K, kernels; CO, cobs; S, silage; MR, partial total mixed ration). Trypsin treated and HPLC purified Cry1Ab protein (100 pg) was included as a positive control (C).

Fig 5. Western blot showing fragments of Cry1Ab protein in GIT digesta (Ru, rumen; LI , large intestine; Ce, cecum) extracts from transgenic and non-transgenic diets (MR) fed cows. Trypsin treated and HPLC purified Cry1Ab protein (100 pg) was included as a positive control (C). Each extract except rumen content contained 100 pg Cry1Ab protein (as measured in ELISA), whereas due to low concentration of Cry1Ab protein in rumen content extract 70 pg Cry1Ab protein was used. In non-transgenic feed and digesta extract respective amount of total protein was used.

Fig 6. Western blot showing Cry1Ab protein fragments in total protein extracts (60 μg) of transgenic (T) and non-transgenic feed (NT) and respective transgenic (T1, T2, T3) and non-transgenic (NT1, NT2, NT3) ration fed feces of dairy cows. Trypsin treated and HPLC purified Cry1Ab protein (100 pg) was used as a positive control (C).

FIGURES

Fig 1.

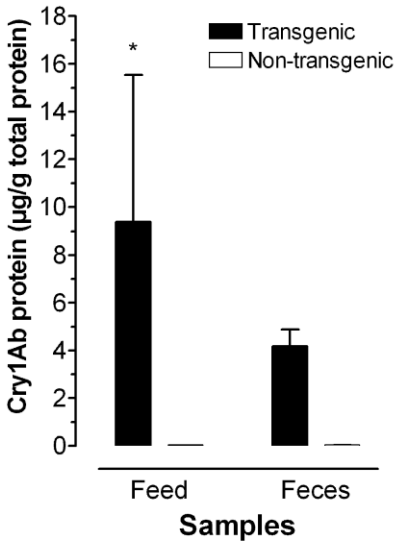

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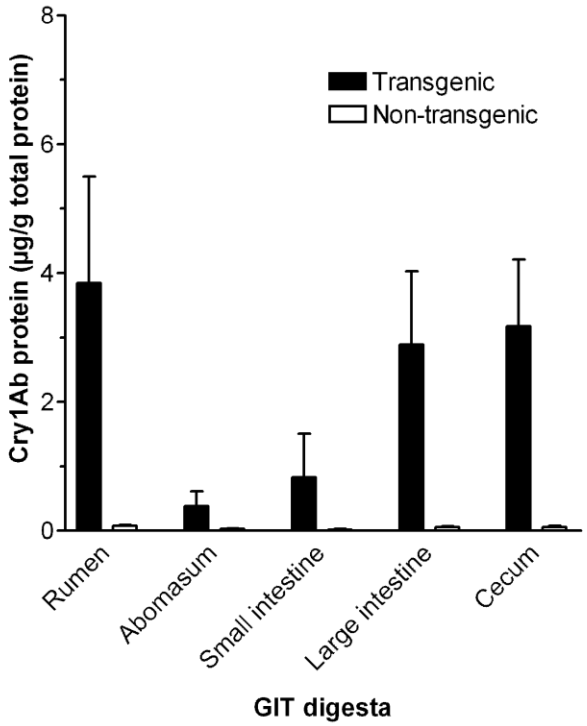
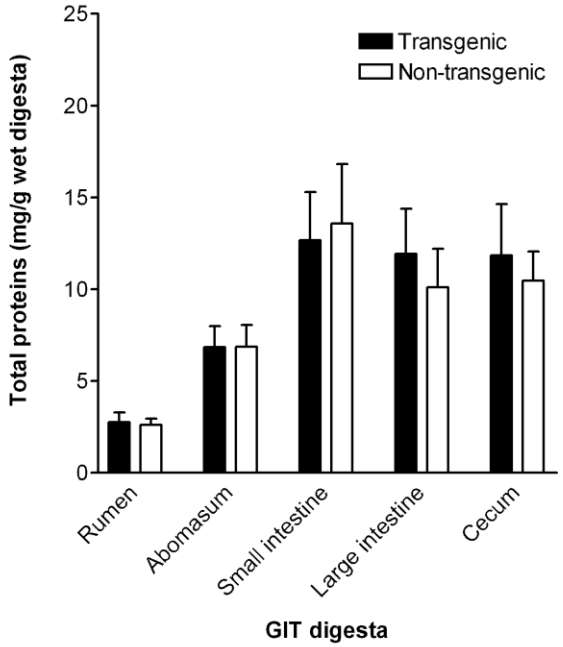
Fig 2.


Transgenic
Non-transgenic
Cry1Ab protein (μg/g total protein)
0
2
4
6
8
Rumen
Abomasum
Small intestine
Large intestine
Cecum
GIT digesta

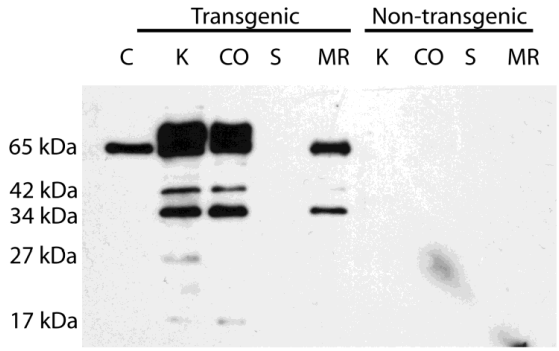
2

Fig 3.


Transgenic
Non-transgenic
Total proteins (mg/g wet digesta)
25
20
15
10
5
0
Rumen
Abomasum
Small intestine
Large intestine
Cecum
GIT digesta

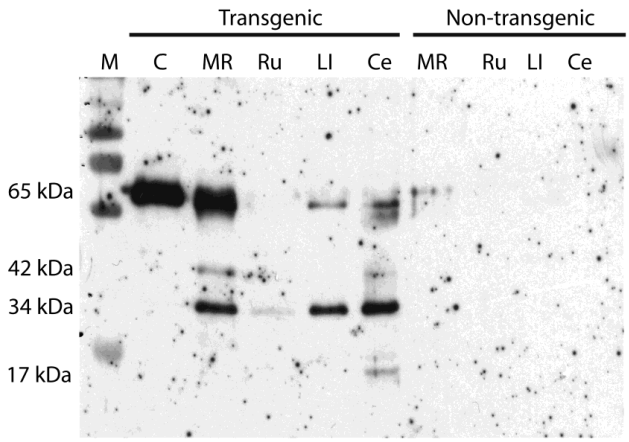
3

Fig 4.


Transgenic
Non-transgenic
C
K
CO
S
MR
K
CO
S
MR
65 kDa
42 kDa
34 kDa
27 kDa
17 kDa

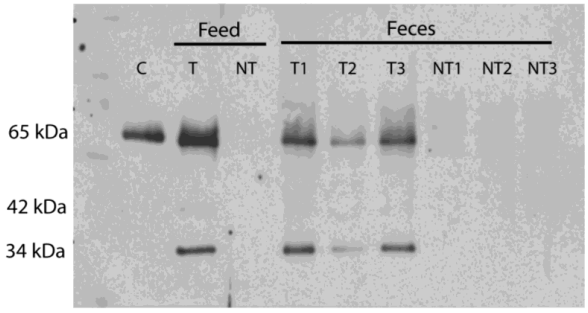
4

Fig 5.


Transgenic
Non-transgenic
M
C
MR
Ru
LI
Ce
MR
Ru
LI
Ce
65 kDa
42 kDa
34 kDa
17 kDa

5

Fig 6.



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